

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> :C07H 21/02, 21/04, C12N 15/11, C12Q  
1/68, C07K 5/00

A1

(11) International Publication Number:

WO 99/40102

(43) International Publication Date:

12 August 1999 (12.08.99)

(21) International Application Number: PCT/US99/02544

(22) International Filing Date: 5 February 1999 (05.02.99)

(30) Priority Data:

09/019,942	6 February 1998 (06.02.98)	US
09/099,041	17 June 1998 (17.06.98)	US
09/207,359	8 December 1998 (08.12.98)	US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications

US	09/207,359 (CON)
Filed on	8 December 1998 (08.12.98)
US	09/099,041 (CON)
Filed on	17 June 1998 (17.06.98)
US	09/019,942 (CON)
Filed on	6 February 1998 (06.02.98)

(71) Applicant (for all designated States except US): MILLENNIUM PHARMACEUTICALS, INC. [US/US]; 640 Memorial Drive, Cambridge, MA 02139 (US).

(72) Inventor; and

(75) Inventor/Applicant (for US only): BERTIN, John [US/US]; Apartment 1, 475 Arsenal Street, Watertown, MA 02172 (US).

(74) Agents: MEIKLEJOHN, Anita, L. et al.; Fish &amp; Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

(54) Title: NOVEL MOLECULES OF THE CARD-RELATED PROTEIN FAMILY AND USES THEREOF

## (57) Abstract

Novel CARD-3, CARD-4L, CARD-4S, CARD-4Y, CARD-4Z, and murine CARD-4L polypeptides, proteins, and nucleic acid molecules are disclosed. In addition to isolated CARD-3, CARD-4L, CARD-4S, CARD-4Y, CARD-4Z, and murine CARD-4L proteins, and the invention further provides CARD-3, CARD-4L, CARD-4S, CARD-4Y, CARD-4Z, and murine CARD-4L fusion proteins, antigenic peptides and anti-CARD-3, anti-CARD-4L and anti-CARD-4S, anti-CARD-4Y, anti-CARD-4Z, and anti-murine CARD-4L antibodies. The invention also provides CARD-3, CARD-4L, CARD-4S, CARD-4Y, CARD-4Z, and murine CARD-4L nucleic acid molecules, recombinant expression vectors containing a nucleic acid molecule of the invention, host cells into which the expression vectors have been introduced and non-human transgenic animals in which a CARD-3, CARD-4L, CARD-4S, CARD-4Y, CARD-4Z, and murine CARD-4L gene has been introduced or disrupted. The invention further provides CARD-3 and CARD-4 target proteins that bind to CARD-3 or CARD-4 and allelic variants of human CARD-4. Diagnostic, screening and therapeutic methods utilizing compositions of the invention are also provided.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						



NOVEL MOLECULES OF THE CARD-RELATED PROTEIN  
FAMILY AND USES THEREOF

5                   Cross Reference to Related Applications

          This application is a continuation-in-part of U.S. Application Serial No. 09/207,359 filed December 8, 1998, which is a continuation-in-part of U.S. Application Serial No. 09/099,041, filed June 17, 1998, which is a  
10 continuation-in-part of U.S. Application Serial No. 09/019,942, filed February 6, 1998. The contents of each of these applications is incorporated herein by this reference.

Background of the Invention

15           In multicellular organisms, homeostasis is maintained by balancing the rate of cell proliferation against the rate of cell death. Cell proliferation is influenced by numerous growth factors and the expression of proto-oncogenes, which typically encourage progression  
20 through the cell cycle. In contrast, numerous events, including the expression of tumor suppressor genes, can lead to an arrest of cellular proliferation.

          In differentiated cells, a particular type of cell death called apoptosis occurs when an internal suicide  
25 program is activated. This program can be initiated by a variety of external signals as well as signals that are generated within the cell in response to, for example, genetic damage. For many years, the magnitude of apoptotic cell death was not appreciated because the  
30 dying cells are quickly eliminated by phagocytes, without an inflammatory response.

          The mechanisms that mediate apoptosis have been intensively studied. These mechanisms involve the activation of endogenous proteases, loss of mitochondrial

- 2 -

function, and structural changes such as disruption of the cytoskeleton, cell shrinkage, membrane blebbing, and nuclear condensation due to degradation of DNA. The various signals that trigger apoptosis are thought to  
5 bring about these events by converging on a common cell death pathway that is regulated by the expression of genes that are highly conserved from worms, such as *C. elegans*, to humans. In fact, invertebrate model systems have been invaluable tools in identifying and  
10 characterizing the genes that control apoptosis. Through the study of invertebrates and more evolved animals, numerous genes that are associated with cell death have been identified, but the way in which their products interact to execute the apoptotic program is poorly  
15 understood.

Caspases, a class of proteins central to the apoptotic program, are cysteine protease having specificity for aspartate at the substrate cleavage site. These proteases are primarily responsible for the  
20 degradation of cellular proteins that lead to the morphological changes seen in cells undergoing apoptosis. For example, one of the caspases identified in humans was previously known as the interleukin-1 $\alpha$  (IL-1 $\alpha$ ) converting enzyme (ICE), a cysteine protease responsible for the  
25 processing of pro-IL-1 $\alpha$  to the active cytokine. Overexpression of ICE in Rat-1 fibroblasts induces apoptosis (Miura et al., Cell 75:653, 1993).

Many caspases and proteins that interact with caspases possess domains of about 60 amino acids called a  
30 caspase recruitment domain (CARD). Hofmann et al. (TIBS 22:155, 1997) and others have postulated that certain apoptotic proteins bind to each other via their CARDS and that different subtypes of CARDS may confer binding specificity, regulating the activity of various caspases,  
35 for example.

- 3 -

The functional significance of CARDS have been demonstrated in recent publications. Duan et al. (Nature 385:86, 1997) showed that deleting the CARD at the N-terminus of RAIDD, a newly identified protein involved in apoptosis, abolished the ability of RAIDD to bind to caspases. In addition, Li et al. (Cell 91:479, 1997) showed that the N-terminal 97 amino acids of apoptotic protease activating factor-1 (Apaf-1) was sufficient to confer caspase-9-binding ability. Inohara et al. (J. Biol. Chem. 273:12296-12300, 1998) showed that Apaf-1 can bind several other caspases such as caspase-4 and caspase-8. Apaf-1 can interact with caspases via CARD-CARD interaction (Li et al., supra, Hu et al., PNAS, 95:4386-4391, 1998).

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) is a transcription factor expressed in many cell types and which activates homologous or heterologous genes that have  $\kappa$ B sites in their promoters. Quiescent NF- $\kappa$ B resides in the cytoplasm as a heterodimer between proteins referred to as p50 and p65 and is complexed with the regulatory protein I $\kappa$ B. NF- $\kappa$ B binding to I $\kappa$ B causes NF- $\kappa$ B to remain in the cytoplasm. At least two dozen stimuli that activate NF- $\kappa$ B are known (New England Journal of Medicine 336:1066, 1997) and they include cytokines, protein kinase C activators, oxidants, viruses, and immune system stimuli. NF- $\kappa$ B activating stimuli activate specific I $\kappa$ B kinases that phosphorylate I $\kappa$ B leading to its degradation. Once liberated from I $\kappa$ B, NF $\kappa$ B translocates to the nucleus and activates genes with  $\kappa$ B sites in their promoters. How all of these NF- $\kappa$ B activating stimuli act is unknown at the present time and it is presumed that novel NF- $\kappa$ B pathway components are involved. NF- $\kappa$ B and the NF- $\kappa$ B pathway has been implicated in mediating chronic inflammation in inflammatory diseases such as asthma, ulcerative colitis, rheumatoid arthritis (New

- 4 -

England Journal of Medicine 336:1066, 1997) and inhibiting NF- $\kappa$ B or NF- $\kappa$ B pathways may be an effective way of treating these diseases. NF- $\kappa$ B and the NF- $\kappa$ B pathway has also been implicated in atherosclerosis  
5 (American Journal of Cardiology 76:18C, 1995), especially in mediating fatty streak formation, and inhibiting NF- $\kappa$ B or NF- $\kappa$ B pathways may be an effective therapy for atherosclerosis.

#### Summary of the Invention

10 The present invention is based, at least in part, on the discovery of genes encoding CARD-3 and CARD-4. The CARD-4 gene can express a long transcript that encodes CARD-4L, a short transcript that encodes partial CARD-4S, or two CARD-4 splice variants. A murine full  
15 length cDNA sequence for the murine ortholog of CARD-4L is also presented. CARD-3 and CARD-4 are intracellular proteins that are predicted to be involved in regulating caspase activation. CARD-4 is found to activate the NF- $\kappa$ B pathway and to enhance caspase 9-mediated cell  
20 death. In addition, proteins that bind to CARD-4 are presented including CARD-3 and hNUDC.

The CARD-3 cDNA described below (SEQ ID NO:1) has a 1620 open reading frame (nucleotides 214 to 1833 of SEQ ID NO:1; SEQ ID NO:3) which encodes a 540 amino acid  
25 protein (SEQ ID NO:2). CARD-3 contains a kinase domain which extends from amino acid 1 to amino acid 300 of SEQ ID NO:2; SEQ ID NO:4, followed by a linker domain at amino acid 301 to amino acid 431 of SEQ ID NO:2; SEQ ID NO:5 and a CARD at amino acid 432 to amino acid 540 of  
30 SEQ ID NO:2; SEQ ID NO:6.

At least four forms of CARD-4 exist in the cell, a long form, CARD-4L, a short form, CARD-4S, and two splice variants, CARD-4Y and CARD-4Z. The cDNA of CARD-4L described below (SEQ ID NO:7) has a 2859 nucleotide open

- 5 -

reading frame (nucleotides 245-3103 of SEQ ID NO:7; SEQ ID NO:9) which encodes a 953 amino acid protein (SEQ ID NO:8). CARD-4L protein possesses a CARD domain (amino acids 15-114; SEQ ID NO:10). The nucleotide sequence of the full length cDNA corresponding to the murine ortholog of human CARD-4L is presented (SEQ ID NO:42) as is the predicted amino acid sequence of murine CARD-4L (SEQ ID NO:43). A comparison between the predicted amino acid sequences of human CARD-4L and murine CARD-4L is also depicted in Figure 17.

Human CARD-4L is also predicted to have a nucleotide binding domain which extends from about amino acid 198 to about amino acid 397 of SEQ ID NO:8; SEQ ID NO:11, a Walker Box "A", which extends from about amino acid 202 to about amino acid 209 of SEQ ID NO:8; SEQ ID NO:12, a Walker Box "B", which extends from about amino acid 280 to about amino acid 284, of SEQ ID NO:8; SEQ ID NO:13, a kinase 1a (P-loop) subdomain, which extends from about amino acid 127 to about amino acid 212 of SEQ ID NO:8; SEQ ID NO:46, a kinase 2 subdomain, which extends from about amino acid 273 to about amino acid 288 of SEQ ID NO:8; SEQ ID NO:47, a kinase 3a subdomain, which extends from about amino acid 327 to about amino acid 338 of SEQ ID NO:8; SEQ ID NO:14, and ten Leucine-rich repeats which extend from about amino acid 674 to about amino acid 950 of SEQ ID NO:8. The first Leucine-rich repeat extends from about amino acid 674 to about amino acid 701 of SEQ ID NO:8; SEQ ID NO:15. The second Leucine-rich repeat extends from about amino acid 702 to about amino acid 727 of SEQ ID NO:8; SEQ ID NO:16. The third Leucine-rich repeat extends from about amino acid 728 to about amino acid 754 of SEQ ID NO:8; SEQ ID NO:17. The fourth Leucine-rich repeat extends from about amino acid 755 to about amino acid 782 of SEQ ID NO:8; SEQ ID NO:18. The fifth Leucine-rich repeat extends from about

- 6 -

amino acid 783 to about amino acid 810 of SEQ ID NO:8;  
SEQ ID NO:19. The sixth Leucine-rich repeat extends from  
about amino acid 811 to about amino acid 838 of SEQ ID  
NO:8; SEQ ID NO:20. The seventh Leucine-rich repeat  
5 extends from about amino acid 839 to about amino acid 866  
of SEQ ID NO:8; SEQ ID NO:21. The eighth Leucine-rich  
repeat extends from about amino acid 867 to about amino  
acid 894 of SEQ ID NO:8; SEQ ID NO:22. The ninth  
Leucine-rich repeat extends from about amino acid 895 to  
10 about amino acid 922 of SEQ ID NO:8; SEQ ID NO:23 and the  
tenth leucine-rich repeat extends from about amino acid  
923 to about amino acid 950 of SEQ ID NO:8; SEQ ID NO:24.

The partial cDNA of CARD-4S described below (SEQ  
ID NO:25) has a 1470 nucleotide open reading frame  
15 (nucleotides 1-1470 of SEQ ID NO:25; SEQ ID NO:27) which  
encodes a 490 amino acid protein (SEQ ID NO:26). CARD-4S  
protein possesses a CARD domain (amino acids 1-74 of SEQ  
ID NO:26; SEQ ID NO:28). CARD-4S is predicted to have a  
P-Loop which extends from about amino acid 163 to about  
20 amino acid 170 of SEQ ID NO:26; SEQ ID NO:29, and a  
Walker Box "B" which extends from about amino acid 241 to  
about amino acid 245 of SEQ ID NO:26; SEQ ID NO:30.

A human CARD-4Y nucleotide cDNA sequence is  
presented (SEQ ID NO:38) as is the amino acid sequence of  
25 the predicted CARD-4Y product (SEQ ID NO:39). A human  
CARD-4Z nucleotide cDNA sequence is presented (SEQ ID  
NO:40) as is the amino acid sequence of the predicted  
CARD-4Z product (SEQ ID NO:41). A comparison of the  
CARD-4Y, CARD-4Z, and human CARD-4L predicted amino acid  
30 sequences is also shown in Figure 14.

Like other proteins containing a CARD domain, both  
CARD-3 and CARD-4 are expected to participate in the  
network of interactions that lead to caspase activity.  
Human CARD-4L is expected to play a functional role in  
35 caspase activation similar to that of Apaf-1 (Zou et al.,

- 7 -

Cell, 90:405-413, 1997). For example, upon activation, CARD-4L might bind a nucleotide, thus allowing CARD-4L to bind and activate a CARD-containing caspase via a CARD-CARD interaction, leading to apoptotic death of the  
5 cell. Accordingly, CARD-3 and CARD-4 molecules are useful as modulating agents in regulating a variety of cellular processes including cell growth and cell death. In one aspect, this invention provides isolated nucleic acid molecules encoding CARD-3 or CARD-4 proteins or  
10 biologically active portions thereof, as well as nucleic acid fragments suitable as primers or hybridization probes for the detection of CARD-3 or CARD-4 encoding nucleic acids.

The invention encompasses methods of diagnosing  
15 and treating patients who are suffering from a disorder associated with an abnormal level or rate (undesirably high or undesirably low) of apoptotic cell death, abnormal activity of the Fas/APO-1 receptor complex, abnormal activity of the TNF receptor complex, or  
20 abnormal activity of a caspase by administering a compound that modulates the expression of CARD-3 or CARD-4 (at the DNA, mRNA or protein level, e.g., by altering mRNA splicing) or by altering the activity of CARD-3 or CARD-4. Examples of such compounds include  
25 small molecules, antisense nucleic acid molecules, ribozymes, and polypeptides.

Certain disorders are associated with an increased number of surviving cells, which are produced and continue to survive or proliferate when apoptosis is  
30 inhibited. These disorders include cancer (particularly follicular lymphomas, carcinomas associated with mutations in p53, and hormone-dependent tumors such as breast cancer, prostate cancer, and ovarian cancer), autoimmune disorders (such as systemic lupus  
35 erythematosus, immune-mediated glomerulonephritis), and

- 8 -

viral infections (such as those caused by herpesviruses, poxviruses, and adenoviruses).

Failure to remove autoimmune cells that arise during development or that develop as a result of somatic  
5 mutation during an immune response can result in autoimmune disease. One of the molecules that plays a critical role in regulating cell death in lymphocytes is the cell surface receptor for Fas.

Populations of cells are often depleted in the  
10 event of viral infection, with perhaps the most dramatic example being the cell depletion caused by the human immunodeficiency virus (HIV). Surprisingly, most T cells that die during HIV infections do not appear to be infected with HIV. Although a number of explanations  
15 have been proposed, recent evidence suggests that stimulation of the CD4 receptor results in the enhanced susceptibility of uninfected T cells to undergo apoptosis.

A wide variety of neurological diseases are  
20 characterized by the gradual loss of specific sets of neurons. Such disorders include Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS) retinitis pigmentosa, spinal muscular atrophy, and various forms of cerebellar degeneration. The cell loss  
25 in these diseases does not induce an inflammatory response, and apoptosis appears to be the mechanism of cell death.

In addition, a number of hematologic diseases are associated with a decreased production of blood cells.  
30 These disorders include anemia associated with chronic disease, aplastic anemia, chronic neutropenia, and the myelodysplastic syndromes. Disorders of blood cell production, such as myelodysplastic syndrome and some forms of aplastic anemia, are associated with increased  
35 apoptotic cell death within the bone marrow. These



- 9 -

disorders could result from the activation of genes that promote apoptosis, acquired deficiencies in stromal cells or hematopoietic survival factors, or the direct effects of toxins and mediators of immune responses.

5 Two common disorders associated with cell death are myocardial infarctions and stroke. In both disorders, cells within the central area of ischemia, which is produced in the event of acute loss of blood flow, appear to die rapidly as a result of necrosis.  
10 However, outside the central ischemic zone, cells die over a more protracted time period and morphologically appear to die by apoptosis.

The invention features a nucleic acid molecule which is at least 45% (or 55%, 65%, 75%, 85%, 95%, or  
15 98%) identical to the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID:25, SEQ ID NO:27, SEQ ID NO:38, SEQ ID NO:40, and SEQ ID NO:42, the nucleotide sequence of the cDNA insert of the plasmid deposited with ATCC as Accession Number (the  
20 "cDNA of ATCC \_\_\_\_\_"), the nucleotide sequence of the cDNA insert of the plasmid deposited with ATCC as Accession Number (the "cDNA of ATCC\_\_\_\_\_"), the nucleotide sequence of the cDNA insert of the plasmid deposited with ATCC as Accession Number (the "cDNA of  
25 ATCC\_\_\_\_\_"), or a complement thereof.

The invention features a nucleic acid molecule which includes a fragment of at least 150 (300, 325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1300, 1600 or 1931) nucleotides of the nucleotide  
30 sequence shown in SEQ ID NO:1, or SEQ ID NO:3, or the nucleotide sequence of the cDNA ATCC \_\_\_\_\_, or a complement thereof.

The invention also features a nucleic acid molecule which includes a fragment of at least 150 (350,  
35 400, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1300,

- 10 -

1600, 1900, 2100, 2400, 2700, 3000, or 3382) nucleotides of the nucleotide sequence shown in SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO: 43 or the nucleotide sequence of the cDNA ATCC \_\_\_\_\_, or a complement thereof.

5       Also within the invention is a nucleic acid molecule which includes a fragment of at least 150 (350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1300, 1600, 1900, 2100, 2400, 2700, and 3080) nucleotides of the nucleotide sequence shown in SEQ ID NO:25, SEQ ID  
10 NO:27, SEQ ID NO:38, SEQ ID NO:40, or the nucleotide sequence of the cDNA ATCC \_\_\_\_\_, or a complement thereof.

      The invention features a nucleic acid molecule which includes a nucleotide sequence encoding a protein  
15 having an amino acid sequence that is at least 45% (or 55%, 65%, 75%, 85%, 95%, or 98%) identical to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:26, SEQ ID NO:39, SEQ ID NO:41, and SEQ ID NO:43, or the amino acid sequence encoded by the cDNA of ATCC \_\_\_\_\_, the  
20 amino acid sequence encoded by the cDNA of ATCC \_\_\_\_\_, or the amino acid sequence encoded by the cDNA of ATCC \_\_\_\_\_.

      In an embodiment, a CARD-3 nucleic acid molecule has the nucleotide sequence shown in SEQ ID NO:1, or SEQ ID NO:3, or the nucleotide sequence of the cDNA of ATCC  
25 \_\_\_\_\_. In another embodiment, a CARD-4L nucleic acid molecule has the nucleotide sequence shown in SEQ ID NO:7, or SEQ ID NO:9, or the nucleotide sequence of the cDNA of ATCC \_\_\_\_\_. In yet another embodiment, a CARD-4S nucleic acid molecule has the nucleotide sequence shown  
30 in SEQ ID NO:25, or SEQ ID NO:27, or the nucleotide sequence of the cDNA of ATCC \_\_\_\_\_. In another embodiment, a murine CARD-4L nucleic acid molecule has the nucleotide sequence shown in SEQ ID NO:42. In another embodiment, a CARD-4Y nucleic acid molecule has

- 11 -

the nucleotide sequence shown in SEQ ID NO:38 or the nucleotide sequence of the cDNA of ATCC \_\_\_\_\_. In another embodiment, a CARD-4Z nucleic acid molecule has the nucleotide sequence shown in SEQ ID NO:40 or the  
5 nucleotide sequence of the cDNA of ATCC \_\_\_\_\_.

Also within the invention is a nucleic acid molecule which encodes a fragment of a polypeptide having the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:8 or SEQ ID NO:26 or SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID  
10 NO:43, the fragment including at least 15 (25, 30, 50, 100, 150, 300, 400 or 540, 600, 700, 800, 953) contiguous amino acids of SEQ ID NO:2 or SEQ ID NO:8 or SEQ ID NO:26 or SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID NO:43 or the polypeptide encoded by the cDNA of ATCC Accession Number \_  
15 \_\_\_\_\_, or the polypeptide encoded by the cDNA of ATCC Accession Number \_\_\_\_\_ or the polypeptide encoded by the cDNA of ATCC Accession Number \_\_\_\_\_.

The invention includes a nucleic acid molecule  
20 which encodes a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID NO:43 or an amino acid sequence encoded by the cDNA of ATCC Accession Number \_\_\_\_\_, wherein the nucleic acid molecule  
25 hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:38 or SEQ ID NO:40 or SEQ ID NO:42 under stringent conditions. The invention also includes a nucleic acid molecule which encodes a naturally occurring allelic variant of a polypeptide  
30 comprising the amino acid sequence of SEQ ID NO:8 or an amino acid sequence encoded by the cDNA of ATCC Accession Number \_\_\_\_\_, wherein the nucleic acid molecule hybridizes to a nucleic acid molecule comprising SEQ ID NO:7 or SEQ ID NO:9 under stringent conditions.

- 12 -

The invention also includes a nucleic acid molecule which encodes a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:26 or an amino acid sequence  
5 encoded by the cDNA of ATCC Accession Number \_\_\_\_\_, wherein the nucleic acid molecule hybridizes to a nucleic acid molecule comprising SEQ ID NO:25 or SEQ ID NO:27 under stringent conditions. In general, an allelic variant of a gene will be readily identifiable as mapping  
10 to the same chromosomal location as said gene. For example, in Example 6, the chromosomal location of the human CARD-4 gene is discovered to be chromosome 7 close to the SHGC-31928 genetic marker. Allelic variants of human CARD-4 will be readily identifiable as mapping to  
15 the human CARD-4 locus on chromosome 7 near genetic marker SHGC-31928.

Also within the invention are: an isolated CARD-3 protein having an amino acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98% identical to  
20 the amino acid sequence of SEQ ID NO:2; an isolated CARD-3 protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the kinase domain of SEQ ID NO:2 (e.g., about amino acid residues 1 to 300 of SEQ ID NO:2; SEQ ID NO:4); and an isolated  
25 CARD-3 protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the linker domain of SEQ ID NO:2 (e.g., about amino acid residues 301 to 431 of SEQ ID NO:2; SEQ ID NO:5); an isolated CARD-3 protein having an amino acid sequence that is at  
30 least about 85%, 95%, or 98% identical to the CARD domain of SEQ ID NO:2 (e.g., about amino acid residues 432 to 540 of SEQ ID NO:2; SEQ ID NO:6); an isolated CARD-4L protein having an amino acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98% identical to  
35 the amino acid sequence of SEQ ID NO:8; an isolated

- 13 -

CARD-4L protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the CARD domain of SEQ ID NO:8 (e.g., about amino acid residues 15 to 114 of SEQ ID NO:8; SEQ ID NO:10); an isolated CARD-4L  
5 protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the nucleotide binding domain of SEQ ID NO:8 (e.g., about amino acid residues 198 to 397 of SEQ ID NO:8; SEQ ID NO:11; an isolated CARD-4L protein having an amino acid sequence  
10 that is at least about 85%, 95%, or 98% identical to the kinase 1a (P-loop) subdomain SEQ ID NO:8 (e.g., about amino acid 127 to about amino acid 212 of SEQ ID NO:8; SEQ ID NO:46); an isolated CARD-4L protein having an amino acid sequence that is at least about 85%, 95%, or  
15 98% identical to the kinase 2 subdomain of SEQ ID NO:8 (e.g., about amino acid 273 to about amino acid 288 of SEQ ID NO:8; SEQ ID NO:47); an isolated CARD-4L protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to a kinase 3a subdomain of SEQ ID  
20 NO:8 (e.g., about amino acid residues 327 to 338 of SEQ ID NO:8; SEQ ID NO:14); an isolated CARD-4L protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the Leucine-rich repeats of SEQ ID NO:8 (e.g., about amino acid residues 674 to 701 of  
25 SEQ ID NO:8; SEQ ID NO:15; from amino acid 702 to amino acid 727 of SEQ ID NO:8; SEQ ID NO:16; which extends from amino acid 728 to amino acid 754 SEQ ID NO:8; SEQ ID NO:17; from amino acid 755 to amino acid 782 of SEQ ID NO:8; SEQ ID NO:18; from amino acid 783 to amino acid 810  
30 of SEQ ID NO:8; SEQ ID NO:19; from amino acid 811 to amino acid 838 of SEQ ID NO:8; SEQ ID NO:20 from amino acid 839 to amino acid 866 of SEQ ID NO:8; SEQ ID NO:21; from amino acid 867 to amino acid 894 of SEQ ID NO:8; SEQ ID NO:22; from amino acid 895 to amino acid 922 of SEQ ID  
35 NO:8; SEQ ID NO:23; and from amino acid 923 to amino acid

- 14 -

950 of SEQ ID NO:8; SEQ ID NO:24); an isolated CARD-4S protein having an amino acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98% identical to the amino acid sequence of SEQ ID NO:26; an isolated

5 CARD-4S protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the CARD domain of SEQ ID NO:26 (e.g., about amino acid residues 1 to 74 of SEQ ID NO:26; SEQ ID NO:28). Also within the invention are: an isolated murine CARD-4L protein having an amino

10 acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98% identical to the amino acid sequence of SEQ ID NO:43. Also within the invention are: an isolated CARD-4Y protein having an amino acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98%

15 identical to the amino acid sequence of SEQ ID NO:39. Also within the invention are: an isolated CARD-4Z protein having an amino acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98% identical to the amino acid sequence of SEQ ID NO:41.

20 Also within the invention are: an isolated CARD-3 protein which is encoded by a nucleic acid molecule having a nucleotide sequence that is at least about 65%, preferably 75%, 85%, or 95% identical to SEQ ID NO:3 or the cDNA of ATCC \_\_\_\_\_; an isolated CARD-3 protein which

25 is encoded by a nucleic acid molecule having a nucleotide sequence at least about 65% preferably 75%, 85%, or 95% identical to the kinase domain encoding portion of SEQ ID NO:1 (e.g., about nucleotides 213 to 1113 of SEQ ID NO:1); an isolated CARD-3 protein which is encoded by a

30 nucleic acid molecule having a nucleotide sequence at least about 65% preferably 75%, 85%, or 95% identical the linker domain encoding portion of SEQ ID NO:1 (e.g., about nucleotides 1114 to 1506 of SEQ ID NO:1); and an isolated CARD-3 protein which is encoded by a nucleic

35 acid molecule having a nucleotide sequence at least about

- 15 -

65% preferably 75%, 85%, or 95% identical the CARD domain encoding portion of SEQ ID NO:1 (e.g., about nucleotides 1507 to 1833 of SEQ ID NO:1); and an isolated CARD-3 protein which is encoded by a nucleic acid molecule  
5 having a nucleotide sequence which hybridizes under stringent hybridization conditions to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:3 or the non-coding strand of the cDNA of ATCC \_\_\_\_\_. Also within the invention are: an isolated CARD-4Y protein  
10 which is encoded by a nucleic acid molecule having a nucleotide sequence that is at least about 65%, preferably 75%, 85%, or 95% identical to SEQ ID NO:38 or the cDNA of ATCC \_\_\_\_\_. Also within the invention are nucleic acid molecules which include about nucleotides  
15 2759 to 2842 of SEQ ID NO:7; about nucleotides 2843 to 2926 of SEQ ID NO:7; about nucleotides 2927 to 3010 of SEQ ID NO:7; about nucleotides 3011 to 3094 of SEQ ID NO:7; and an isolated CARD-4L protein which is encoded by a nucleic acid molecule having a nucleotide sequence  
20 which hybridizes under stringent hybridization conditions to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:9 or the non-coding strand of the cDNA of ATCC \_\_\_\_\_.

Also within the invention are: an isolated CARD-4S  
25 protein which is encoded by a nucleic acid molecule having a nucleotide sequence that is at least about 65%, preferably 75%, 85%, or 95% identical to SEQ ID NO:27 or the cDNA of ATCC \_\_\_\_\_; an isolated CARD-3 protein which is encoded by a nucleic acid molecule having a nucleotide  
30 sequence at least about 65% preferably 75%, 85%, or 95% identical the CARD domain encoding portion of SEQ ID NO:25 (e.g., about nucleotides 1 to 222 of SEQ ID NO:25); an isolated CARD-3 protein which is encoded by a nucleic acid molecule having a nucleotide sequence at least about  
35 65% preferably 75%, 85%, or 95% identical the P-Loop

- 16 -

encoding portion of SEQ ID NO:25 (e.g., about nucleotides 485 to 510 of SEQ ID NO:25).

Also within the invention is a polypeptide which is a naturally occurring allelic variant of a polypeptide that includes the amino acid sequence of SEQ ID NO:2 or an amino acid sequence encoded by the cDNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, wherein the polypeptide is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 or SEQ ID NO:3 under stringent conditions.

Also within the invention is a polypeptide which is a naturally occurring allelic variant of a polypeptide that includes the amino acid sequence of SEQ ID NO:8 or an amino acid sequence encoded by the cDNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, wherein the polypeptide is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:7 or SEQ ID NO:9 under stringent conditions.

Also within the invention is a polypeptide which is a naturally occurring allelic variant of a polypeptide that includes the amino acid sequence of SEQ ID NO:26 or an amino acid sequence encoded by the cDNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, wherein the polypeptide is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:25 or SEQ ID NO:27 under stringent conditions.

Another embodiment of the invention features CARD-3 or CARD-4 nucleic acid molecules which specifically detect CARD-3 or CARD-4 nucleic acid molecules, relative to nucleic acid molecules encoding other members of the CARD superfamily. For example, in one embodiment, a CARD-4L nucleic acid molecule



- 17 -

hybridizes under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:7, SEQ ID NO:9, or the cDNA of ATCC \_\_\_\_\_, or a complement thereof. In another embodiment, the CARD-4L  
5 nucleic acid molecule is at least 300 (350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1300, 1600, 1900, 2100, 2400, 2700, 3000, or 3382) nucleotides in length and hybridizes under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence  
10 shown in SEQ ID NO:7, SEQ ID NO:9, the cDNA of ATCC \_\_\_\_\_, or a complement thereof. In another embodiment, an isolated CARD-4L nucleic acid molecule comprises nucleotides 287 to 586 of SEQ ID NO:7, encoding the CARD domain of CARD-4L, or a complement thereof. In yet  
15 another embodiment, the invention provides an isolated nucleic acid molecule which is antisense to the coding strand of a CARD-4L nucleic acid.

Another aspect of the invention provides a vector, e.g., a recombinant expression vector, comprising a  
20 CARD-3 or a CARD-4L nucleic acid molecule of the invention. In another embodiment the invention provides a host cell containing such a vector. The invention also provides a method for producing CARD-3 or CARD-4 protein by culturing, in a suitable medium, a host cell of the  
25 invention containing a recombinant expression vector such that a CARD-3 or CARD-4 protein is produced.

Another aspect of this invention features isolated or recombinant CARD-3 or CARD-4 proteins and polypeptides. Preferred CARD-3 or CARD-4 proteins and  
30 polypeptides possess at least one biological activity possessed by naturally occurring human CARD-3 or CARD-4, e.g., (1) the ability to form protein:protein interactions with proteins in the apoptotic signalling pathway; (2) the ability to form  
35 CARD-CARD interactions with proteins in the apoptotic

- 18 -

signaling pathway; (3) the ability to bind the CARD-3 or CARD-4 ligand; (4) and the ability to bind to an intracellular target. Other activities include: (1) modulation of cellular proliferation, (2) modulation of cellular differentiation and (3) modulation of cellular death (4) modulation of the NF- $\kappa$ B pathway.

The CARD-3 or CARD-4 proteins of the present invention, or biologically active portions thereof, can be operatively linked to a non-CARD-3 or non-CARD-4 polypeptide (e.g., heterologous amino acid sequences) to form CARD-3 or CARD-4 fusion proteins, respectively. The invention further features antibodies that specifically bind CARD-3 or CARD-4 proteins, such as monoclonal or polyclonal antibodies. In addition, the CARD-3 or CARD-4 proteins or biologically active portions thereof can be incorporated into pharmaceutical compositions, which optionally include pharmaceutically acceptable carriers.

In another aspect, the present invention provides a method for detecting the presence of CARD-3 or CARD-4 activity or expression in a biological sample by contacting the biological sample with an agent capable of detecting an indicator of CARD-3 or CARD-4 activity such that the presence of CARD-3 or CARD-4 activity is detected in the biological sample.

In another aspect, the invention provides a method for modulating CARD-3 or CARD-4 activity comprising contacting a cell with an agent that modulates (inhibits or stimulates) CARD-3 or CARD-4 activity or expression such that CARD-3 or CARD-4 activity or expression in the cell is modulated. In one embodiment, the agent is an antibody that specifically binds to CARD-3 or CARD-4 protein. In another embodiment, the agent modulates expression of CARD-3 or CARD-4 by modulating transcription of a CARD-3 or CARD-4 gene, splicing of a

- 19 -

CARD-3 or CARD-4 mRNA, or translation of a CARD-3 or CARD-4 mRNA. In yet another embodiment, the agent is a nucleic acid molecule having a nucleotide sequence that is antisense to the coding strand of the CARD-3 or CARD-4 mRNA or the CARD-3 or CARD-4 gene.

In one embodiment, the methods of the present invention are used to treat a subject having a disorder characterized by aberrant CARD-3 or CARD-4 protein or nucleic acid expression or activity by administering an agent which is a CARD-3 or CARD-4 modulator to the subject. In one embodiment, the CARD-3 or CARD-4 modulator is a CARD-3 or CARD-4 protein. In another embodiment the CARD-3 or CARD-4 modulator is a CARD-3 or CARD-4 nucleic acid molecule. In other embodiments, the CARD-3 or CARD-4 modulator is a peptide, peptidomimetic, or other small molecule.

The present invention also provides a diagnostic assay for identifying the presence or absence of a genetic lesion or mutation characterized by at least one of: (i) aberrant modification or mutation of a gene encoding a CARD-3 or CARD-4 protein; (ii) mis-regulation of a gene encoding a CARD-3 or CARD-4 protein; (iii) aberrant RNA splicing; and (iv) aberrant post-translational modification of a CARD-3 or CARD-4 protein, wherein a wild-type form of the gene encodes a protein with a CARD-3 or CARD-4 activity.

In another aspect, the invention provides a method for identifying a compound that binds to or modulates the activity of a CARD-3 or CARD-4 protein. In general, such methods entail measuring a biological activity of a CARD-3 or CARD-4 protein in the presence and absence of a test compound and identifying those compounds which alter the activity of the CARD-3 or CARD-4 protein.

- 20 -

The invention also features methods for identifying a compound which modulates the expression of CARD-3 or CARD-4 by measuring the expression of CARD-3 or CARD-4 in the presence and absence of a compound.

5 Other features and advantages of the invention will be apparent from the following detailed description and claims.

#### Brief Description of the Drawings

Figure 1 depicts the cDNA sequence (SEQ ID NO:1) of human CARD-3. The open reading frame of CARD-3 (SEQ ID NO:1) extends from nucleotide 213 to nucleotide 1833 nucleotide (SEQ ID NO:3).

Figure 2 depicts the predicted amino acid sequence (SEQ ID NO:2) of human CARD-3.

15 Figure 3 depicts the cDNA sequence (SEQ ID NO:7) of CARD-4L. The open reading frame of SEQ ID NO:7 extends from nucleotide 245 to nucleotide 3103 (SEQ ID NO:9).

Figure 4 depicts the predicted amino acid sequence (SEQ ID NO:8) of human CARD-4L.

Figure 5 depicts the partial cDNA sequence (SEQ ID NO:25) of CARD-4S and the predicted amino acid sequence (SEQ ID NO:25) of human CARD-4S. The open reading frame of CARD-4 (SEQ ID NO:25) extends from nucleotide 1 to 25 nucleotide 1470 (SEQ ID NO:27).

Figure 6 depicts the predicted amino acid sequence (SEQ ID NO:26) of human CARD-4S.

Figure 7 depicts an alignment of the CARD domains of CARD-4 (SEQ ID NO:10), CARD-3 (SEQ ID NO:6), ARC-CARD (SEQ ID NO:31), cIAP1-CARD (SEQ ID NO:32) and cIAP2-CARD (SEQ ID NO:33).

- 21 -

Figure 8 is a plot showing predicted structural features of human CARD-4L.

Figure 9 is a plot showing predicted structural features of human CARD-4S.

5        Figure 10 depicts the cDNA sequence (SEQ ID NO:38) of the human CARD-4Y splice variant clone. The predicted open reading frame of the human CARD-4Y splice variant clone extends from nucleotide 438 to nucleotide 1184.

10       Figure 11 depicts the amino acid sequence (SEQ ID NO:39) of the protein predicted to be encoded by the human CARD-4Y cDNA open reading frame.

15       Figure 12 depicts the cDNA sequence (SEQ ID NO:40) of the human CARD-4Z splice variant clone. The predicted open reading frame of the human CARD-4Z splice variant clone extends from nucleotide 489 to nucleotide 980.

Figure 13 depicts the amino acid sequence (SEQ ID NO:41) of the protein predicted to be encoded by the human CARD-4Z cDNA open reading frame.

20       Figure 14 depicts an alignment of human CARD-4L (SEQ ID NO:8), the predicted amino acid sequence of human CARD-4Y (SEQ ID NO:39), and the predicted amino acid sequence of human CARD-4Z (SEQ ID NO:41).

Figure 15 depicts the nucleotide sequence of the murine CARD-4L cDNA (SEQ ID NO:42).

25       Figure 16 depicts the predicted amino acid sequence of murine CARD-4L (SEQ ID NO:43).

Figure 17 depicts an alignment of human CARD-4L (SEQ ID NO:8) and the predicted amino acid sequence of murine CARD-4L (SEQ ID NO:43).

30       Figure 18 depicts a 32042 nucleotide genomic sequence of CARD-4.

#### Detailed Description of the Invention

The present invention is based, in part, on the discovery of cDNA molecules encoding human CARD-3, human

- 22 -

CARD-4 and partial murine CARD-4L proteins. A nucleotide sequence encoding a human CARD-3 protein is shown in Figure 1 (SEQ ID NO:1; SEQ ID NO:3 includes the open reading frame only). A predicted amino acid sequence of CARD-3 protein is also shown in Figure 2 (SEQ ID NO:2). CARD-4 has at least two forms, a long form, CARD-4L, and a short form, CARD-4S, as well as two or more splice variants. A nucleotide sequence encoding a human CARD-4L protein is shown in Figure 3 (SEQ ID NO:7; SEQ ID NO:9 includes the open reading frame only). A predicted amino acid sequence of CARD-4L protein is also shown in Figure 4 (SEQ ID NO:8). A nucleotide sequence encoding a human CARD-4S protein is shown in Figure 5 (SEQ ID NO:25; SEQ ID NO:27 includes the open reading frame only). A predicted amino acid sequence of CARD-4S protein is also shown in Figure 6 (SEQ ID NO:26). Two additional splice variants of human CARD-4 are provided in Figures 10 and 11 (human CARD-4Y) and Figures 12 and 13 (human CARD-4Z) (predicted amino acid sequences: SEQ ID NO:39 and SEQ ID NO:41 and nucleic acid sequences: SEQ ID NO:38 and SEQ ID NO:40). These two splice variants are predicted to contain 249 and 164 amino acids, respectively. An alignment of human CARD-4Y, human CARD-4Z and human CARD-4L is shown in Figure 14.

In addition to the human CARD-4 proteins, a full length nucleotide sequence of the murine ortholog of human CARD-4L is provided in Figure 15 (SEQ ID NO:42). An alignment of murine CARD-4L with human CARD-4L is shown in Figure 17.

The human CARD-3 cDNA of Figure 1 (SEQ ID NO:1), which is approximately 1931 nucleotides long including untranslated regions, encodes a protein amino acid having a molecular weight of approximately 61 kDa (excluding post-translational modifications). A plasmid containing a cDNA encoding human CARD-3 (with the cDNA insert name

- 23 -

of) was deposited with American Type Culture Collection (ATCC), Manassass, VA on \_\_\_\_ and assigned Accession Number \_\_\_\_\_. This deposit will be maintained under the terms of the Budapest Treaty on the International  
5 Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. This deposit was made merely as a convenience for those of skill in the art and is not an admission that a deposit is required under 35 U.S.C. §112.

10           The human CARD-4L cDNA of Figure 3 (SEQ ID NO:7), which is approximately 3382 nucleotides long including untranslated regions, encodes a protein amino acid having a molecular weight of approximately 108 kDa (excluding post-translational modifications). A plasmid containing  
15 a cDNA encoding human CARD-4L (with the cDNA insert name of \_\_\_\_\_) was deposited with American Type Culture Collection (ATCC), Manassass, VA on \_\_\_\_ and assigned Accession Number \_\_\_\_\_. This deposit will be maintained under the terms of the Budapest Treaty on the  
20 International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. This deposit was made merely as a convenience for those of skill in the art and is not an admission that a deposit is required under 35 U.S.C. §112.

25           The human CARD-4S cDNA of Figure 5 (SEQ ID NO:25), which is 3082 nucleotides long including untranslated regions. A plasmid containing a cDNA encoding human CARD-4S (with the cDNA insert name of \_\_\_\_\_) was deposited with American Type Culture Collection (ATCC),  
30 Manassass, VA on \_\_\_\_ and assigned Accession Number \_\_\_\_\_. This deposit will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. This deposit was made merely as a convenience

- 24 -

for those of skill in the art and is not an admission that a deposit is required under 35 U.S.C. §112.

A region of human CARD-4L protein (SEQ ID NO:8) bears some similarity to a CARD domain of CARD-3 (SEQ ID NO:6), ARC-CARD (SEQ ID NO:31), cIAP1-CARD (SEQ ID NO:32), and cIAP2-CARD (SEQ ID NO:33). This comparison is depicted in Figure 7.

Human CARD-3 or CARD-4 are members of a family of molecules (the "CARD family") having certain conserved structural and functional features. The term "family" when referring to the protein and nucleic acid molecules of the invention is intended to mean two or more proteins or nucleic acid molecules having a common structural domain and having sufficient amino acid or nucleotide sequence identity as defined herein. Such family members can be naturally occurring and can be from either the same or different species. For example, a family can contain a first protein of human origin and a homologue of that protein of murine origin, as well as a second, distinct protein of human origin and a murine homologue of that protein. Members of a family may also have common functional characteristics.

In one embodiment, a CARD-3 or CARD-4 protein includes a CARD domain having at least about 65%, preferably at least about 75%, and more preferably about 85%, 95%, or 98% amino acid sequence identity to the CARD domain of SEQ ID NO:6 or the CARD domain of SEQ ID NO:10 or the CARD domain of SEQ ID NO:28.

Preferred CARD-3 or CARD-4 polypeptides of the present invention have an amino acid sequence sufficiently identical to the CARD domain consensus amino acid sequence of SEQ ID NO:6, SEQ ID NO:10, SEQ ID NO:28, respectively. The CARD-3 polypeptide also has an amino acid sequence sufficiently identical to the kinase domain consensus sequence of SEQ ID NO:4, and an amino acid



- 25 -

sequence that is sufficiently identical to the linker domain of SEQ ID NO:5. The CARD-4L polypeptide has an amino acid sequence sufficiently identical to the nucleotide binding domain of SEQ ID NO:11, an amino acid sequence sufficiently identical to the Walker Box "A" of SEQ ID NO:12 or Walker Box "B" of SEQ ID NO:13, an amino acid sequence sufficiently identical to the kinase 1a subdomain of SEQ ID NO:46, an amino acid sequence sufficiently identical to the kinase 2 subdomain of SEQ ID NO:47, or an amino acid sequence sufficiently identical to the kinase 3a subdomain of SEQ ID NO:14, or an amino acid sequence sufficiently identical to the Leucine-rich repeats of SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, and SEQ ID NO:23. As used herein, the term "sufficiently identical" refers to a first amino acid or nucleotide sequence which contains a sufficient or minimum number of identical or equivalent (e.g., an amino acid residue which has a similar side chain) amino acid residues or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences have a common structural domain and/or common functional activity. For example, amino acid or nucleotide sequences which contain a common structural domain having about 65% identity, preferably 75% identity, more preferably 85%, 95%, or 98% identity are defined herein as sufficiently identical.

As used interchangeably herein a "CARD-3 or CARD-4 activity", "biological activity of CARD-3 or CARD-4" or "functional activity of CARD-3 or CARD-4", refers to an activity exerted by a CARD-3 or CARD-4 protein, polypeptide or nucleic acid molecule on a CARD-3 or CARD-4 responsive cell as determined in vivo, or in vitro, according to standard techniques. A CARD-3 or

- 26 -

CARD-4 activity can be a direct activity, such as an association with or an enzymatic activity on a second protein or an indirect activity, such as a cellular signaling activity mediated by interaction of the CARD-3 or CARD-4 protein with a second protein. In an embodiment, a CARD-3 or CARD-4 activity includes at least one or more of the following activities: (i) interaction with proteins in the apoptotic signalling pathway (ii) interaction with a CARD-3 or CARD-4 ligand; or (iii) interaction with an intracellular target protein; (iv) indirect interaction with caspases. For example, in Example 4, CARD-3-containing proteins were shown to associate with CARD-4-containing proteins. In example 9, CARD-4 proteins were shown to induce NF- $\kappa$ B-mediated transcription. In example 10, CARD-3 and CARD-4 were shown to enhance caspase 9 activity.

Accordingly, another embodiment of the invention features isolated CARD-3 or CARD-4 proteins and polypeptides having a CARD-3 or CARD-4 activity.

Various aspects of the invention are described in further detail in the following subsections.

#### **I. Isolated Nucleic Acid Molecules**

One aspect of the invention pertains to isolated nucleic acid molecules that encode CARD-3 or CARD-4 proteins or biologically active portions thereof, as well as nucleic acid molecules sufficient for use as hybridization probes to identify CARD-3 or CARD-4-encoding nucleic acids (e.g., CARD-3 or CARD-4 mRNA) and fragments for use as PCR primers for the amplification or mutation of CARD-3 or CARD-4 nucleic acid molecules. As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA) and analogs of the DNA or RNA generated using nucleotide

- 27 -

analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

An "isolated" nucleic acid molecule is one which  
5 is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) which naturally flank the nucleic acid (i.e., sequences located  
10 at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated CARD-3 or CARD-4L/S nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5  
15 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular  
20 material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

A nucleic acid molecule of the present invention, e.g., a nucleic acid molecule having the nucleotide  
25 sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, the cDNA of ATCC \_\_\_\_\_, the cDNA of ATCC \_\_\_\_\_, or a complement of any of these nucleotide sequences, can be isolated using standard molecular  
30 biology techniques and the sequence information provided herein. Using all or portion of the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, the cDNA of ATCC \_\_\_\_\_ or the cDNA  
35 of ATCC \_\_\_\_\_, as a hybridization probe, CARD-3 or CARD-4

- 28 -

nucleic acid molecules can be isolated using standard hybridization and cloning techniques (e.g., as described in Sambrook et al., eds., Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

A nucleic acid of the invention can be amplified using cDNA, mRNA or genomic DNA as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to CARD-3 or CARD-4 nucleotide sequences can be prepared by standard synthetic techniques, e.g., using an automated DNA synthesizer.

In another embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule which is a complement of the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, the cDNA of ATCC \_\_\_\_\_ or the cDNA of ATCC \_\_\_\_\_ or a portion thereof. A nucleic acid molecule which is complementary to a given nucleotide sequence is one which is sufficiently complementary to the given nucleotide sequence that it can hybridize to the given nucleotide sequence thereby forming a stable duplex.

Moreover, the nucleic acid molecule of the invention can comprise only a portion of a nucleic acid sequence encoding CARD-3 or CARD-4, for example, a fragment which can be used as a probe or primer or a fragment encoding a biologically active portion of CARD-3 or CARD-4. The nucleotide sequence determined from the cloning of the human CARD-3 or CARD-4, and the partial

- 29 -

murine CARD-4 gene allows for the generation of probes and primers designed for use in identifying and/or cloning CARD-3 or CARD-4 homologues in other cell types, e.g., from other tissues, as well as CARD-3 or CARD-4 homologues and orthologs from other mammals. The probe/primer typically comprises substantially purified oligonucleotide. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12, preferably about 25, more preferably about 50, 75, 100, 125, 150, 175, 200, 250, 300, 350 or 400 consecutive nucleotides of the sense or anti-sense sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, the cDNA of ATCC \_\_\_\_\_, the cDNA of ATCC \_\_\_\_\_ or of a naturally occurring mutant of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, the cDNA of ATCC \_\_\_\_\_, or the cDNA of ATCC \_\_\_\_\_.

Probes based on the human CARD-3 or human CARD-4 or murine CARD-4 nucleotide sequence can be used to detect transcripts or genomic sequences encoding the same or identical proteins. The probe comprises a label group attached thereto, e.g., a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used as a part of a diagnostic test kit for identifying allelic variants and orthologs of the CARD-3 and CARD-4 proteins of the present invention, identifying cells or tissue which mis-express a CARD-3 or CARD-4 protein, such as by measuring a level of a CARD-3 or CARD-4-encoding nucleic acid in a sample of cells from a subject, e.g., detecting CARD-3 or CARD-4 mRNA levels or determining whether a genomic CARD-3 or CARD-4 gene has been mutated or deleted.

A nucleic acid fragment encoding a "biologically active portion of CARD-3 or CARD-4L" can be prepared by isolating a portion of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, or the nucleotide sequence of the cDNA

- 30 -

of ATCC \_\_\_\_\_, or the nucleotide sequence of the cDNA of ATCC \_\_\_\_\_ which encodes a polypeptide having a CARD-3 or CARD-4 biological activity, expressing the encoded portion of CARD-3 or CARD-4 protein (e.g., by recombinant expression in vitro) and assessing the activity of the encoded portion of CARD-3 or CARD-4. For example, a nucleic acid fragment encoding a biologically active portion of CARD-3 or CARD-4 includes a CARD domain, e.g., SEQ ID NO:6 and SEQ ID NO:10 or SEQ ID NO:26.

10 The invention further encompasses nucleic acid molecules that differ from the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, the cDNA of ATCC \_\_\_\_\_ or the cDNA of ATCC \_\_\_\_\_ due to degeneracy of the genetic code and thus encode the same CARD-3 or CARD-4 protein as that encoded by the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, the cDNA of ATCC \_\_\_\_\_ or the cDNA of ATCC \_\_\_\_\_.

20 In addition to the human CARD-3 or CARD-4 nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:38, SEQ ID NO:40, the cDNA of ATCC \_\_\_\_\_, the cDNA of ATCC \_\_\_\_\_, or the cDNA of ATCC \_\_\_\_\_, and the murine CARD-4L cDNA sequence shown in SEQ ID NO:42 it will be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequences of CARD-3 or CARD-4 may exist within a population (e.g., the human population). Such genetic polymorphism in the CARD-3 or CARD-4 gene may exist among individuals within a population due to natural allelic variation. As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules comprising an open reading frame encoding a CARD-3 or

- 31 -

CARD-4 protein, preferably a mammalian CARD-3 or CARD-4 protein. Such natural allelic variations can typically result in 1-5% variance in the nucleotide sequence of the CARD-3 or CARD-4 gene. Any and all such nucleotide variations and resulting amino acid polymorphisms in CARD-3 or CARD-4 that are the result of natural allelic variation and that do not alter the functional activity of CARD-3 or CARD-4 are intended to be within the scope of the invention.

Moreover, nucleic acid molecules encoding CARD-3 or CARD-4 proteins from other species (CARD-3 or CARD-4 orthologs/homologues), which have a nucleotide sequence which differs from that of a human CARD-3 or CARD-4, are intended to be within the scope of the invention. For example, Example 5 describes the murine CARD-4 ortholog. Nucleic acid molecules corresponding to natural allelic variants and homologues of the CARD-3 or CARD-4 cDNA of the invention can be isolated based on their identity to the human CARD-3 or human or murine CARD-4 nucleic acids disclosed herein using the human or murine cDNAs, or a portion thereof, as a hybridization probe according to standard hybridization techniques under stringent hybridization conditions. In general, an allelic variant of a gene will be readily identifiable as mapping to the same chromosomal location as said gene. For example, in Example 6, the chromosomal location of the human CARD-4 gene is discovered to be chromosome 7 close to the SHGC-31928 genetic marker. Allelic variants of human CARD-4 will be readily identifiable as mapping to the human CARD-4 locus on chromosome 7 near genetic marker SHGC-31928.

Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention is at least 300 (325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1300, 1600 or 1931) nucleotides in length

- 32 -

and hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence, preferably the coding sequence, of SEQ ID NO:1, SEQ ID NO:3, or the cDNA of ATCC \_\_\_\_\_. In yet another embodiment, an isolated nucleic acid molecule of the invention is at least 300 (325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, or 1300, 1640, 1900, 2200, 2500, 2800, 3100, or 3382) nucleotides in length and hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence, preferably the coding sequence, of SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:38, SEQ ID NO:40, or the cDNA of ATCC \_\_\_\_\_. Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention is at least 300 (325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, or 1300, 1640, 1900, 2200, 2500, 2800, 3100, 3300, 3600, 3900, 4200 or 4209) nucleotides in length and hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence, preferably the coding sequence, of SEQ ID NO:42.

As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 60% (65%, 70%, preferably 75%) identical to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. An, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C. Preferably, an isolated nucleic acid molecule of the invention that hybridizes under stringent conditions to the sequence of SEQ ID NO:1, SEQ ID NO:3, the cDNA of



- 33 -

ATCC \_\_\_\_\_ corresponds to a naturally-occurring nucleic acid molecule. As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (e.g.,  
5 encodes a natural protein).

In addition to naturally-occurring allelic variants of the CARD-3 or CARD-4 sequence that may exist in the population, the skilled artisan will further appreciate that changes can be introduced by mutation  
10 into the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, the cDNA of ATCC \_\_\_\_\_, the cDNA of ATCC \_\_\_\_\_, or the cDNA of ATCC \_\_\_\_\_, thereby leading to changes in the amino acid  
15 sequence of the encoded CARD-3, CARD-4L/S protein, CARD-4 splice variant, or murine CARD-4 without altering the functional ability of the CARD-3, CARD-4L/S, CARD-4 splice variant, or murine CARD-4 protein. For example, one can make nucleotide substitutions leading to amino  
20 acid substitutions at "non-essential" amino acid residues. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of CARD-3, CARD-4L/S, CARD-4 splice variant, or murine CARD-4 protein (e.g., the sequence of SEQ ID NO:2, SEQ ID  
25 NO:8, SEQ ID NO:26, SEQ ID NO:39, SEQ ID NO:41 and SEQ ID NO:43) without altering the biological activity, whereas an "essential" amino acid residue is required for biological activity. For example, amino acid residues that are conserved among the CARD-3, CARD-4L/S, CARD-4  
30 splice variant, or murine CARD-4 proteins of various species are predicted to be particularly unamenable to alteration.

For example, preferred CARD-3 or CARD-4 proteins of the present invention, contain at least one CARD  
35 domain. Additionally, a CARD-3 protein also contains at

- 34 -

least one kinase domain or at least one linker domain. A CARD domain contains at least one nucleotide binding domain or Leucine-rich repeats. Such conserved domains are less likely to be amenable to mutation. Other amino acid residues, however, (e.g., those that are not conserved or only semi-conserved among CARD-3 or CARD-4 of various species) may not be essential for activity and thus are likely to be amenable to alteration.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding CARD-3 or CARD-4 proteins that contain changes in amino acid residues that are not essential for activity. Such CARD-3 or CARD-4 proteins differ in amino acid sequence from SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:25, SEQ ID NO:39, SEQ ID NO:41, or SEQ ID NO:43 and yet retain biological activity. In one embodiment, the isolated nucleic acid molecule includes a nucleotide sequence encoding a protein that includes an amino acid sequence that is at least about 45% identical, 65%, 75%, 85%, 95%, or 98% identical to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:26, SEQ ID NO:39, SEQ ID NO:41 or SEQ ID NO:43.

An isolated nucleic acid molecule encoding a CARD-3 or CARD-4 proteins having a sequence which differs from that of SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:26, SEQ ID NO:39, SEQ ID NO:41 or SEQ ID NO:43, can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of CARD-3 (SEQ ID NO:1, SEQ ID NO:3, the cDNA of ATCC \_\_\_\_\_) or CARD-4L (SEQ ID NO:7, SEQ ID NO:9, the cDNA of ATCC \_\_\_\_\_), or CARD-4S (SEQ ID NO:25, SEQ ID NO:27, the cDNA of ATCC \_\_\_\_\_), or human CARD-4 splice variants (SEQ ID NO:38, SEQ ID NO:40, the cDNA of ATCC \_\_\_\_\_ or the cDNA of ATCC \_\_\_\_\_), or murine CARD-4 (SEQ ID NO:42) such that one or more amino acid substitutions, additions or

- 35 -

deletions are introduced into the encoded protein. Mutations can be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid  
5 substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having  
10 similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine,  
15 serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine,  
20 phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in CARD-3 or CARD-4 is preferably replaced with another amino acid residue from the same side chain family. Alternatively, mutations can be introduced randomly along all or part of a CARD-3 or  
25 CARD-4 coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for CARD-3 or CARD-4 biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed recombinantly and the  
30 activity of the protein can be determined.

In an embodiment, a mutant CARD-3 or CARD-4 protein can be assayed for: (1) the ability to form protein:protein interactions with proteins in the apoptotic signalling pathway; (2) the ability to bind a  
35 CARD-3 or CARD-4 ligand; or (3) the ability to bind to an

- 36 -

intracellular target protein. For example, (1) in Example 7, a two-hybrid screening assay for the physical interaction of CARD-3 and CARD-4 is shown, (2) in Example 8, a two-hybrid system assay for the interaction between CARD-4 and its ligand hNUDC is described, and (3) in Example 12, a coimmunoprecipitation assay for the interaction of CARD-3 with its ligand CARD-4 is shown. In yet another embodiment, a mutant CARD-3 or CARD-4 protein can be assayed for the ability to modulate cellular proliferation, cellular differentiation, or cellular death. For example, in Example 10, assays for the regulation of cellular death (apoptosis) by CARD-3 or CARD-4 are described. In yet another embodiment, a mutant CARD-3 or CARD-4 protein can be assayed for regulation of a cellular signal transduction pathway. For example, in Example 9, an assay for the regulation by CARD-4 of the NF- $\kappa$ B pathway is described.

The present invention encompasses antisense nucleic acid molecules, i.e., molecules which are complementary to a sense nucleic acid encoding a protein, e.g., complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense nucleic acid can be complementary to an entire CARD-3 or CARD-4 coding strand, or to only a portion thereof, e.g., all or part of the protein coding region (or open reading frame). An antisense nucleic acid molecule can be antisense to a noncoding region of the coding strand of a nucleotide sequence encoding CARD-3 or CARD-4. The noncoding regions ("5' and 3' untranslated regions") are the 5' and 3' sequences which flank the coding region and are not translated into amino acids.

Given the coding strand sequences encoding CARD-3 or CARD-4 disclosed herein (e.g., SEQ ID NO:1, SEQ ID

- 37 -

NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:38, SEQ ID NO:40, or SEQ ID NO:42), antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing.

5 The antisense nucleic acid molecule can be complementary to the entire coding region of CARD-3 or CARD-4L/S mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of CARD-3 or CARD-4 mRNA. For example, the

10 antisense oligonucleotide can be complementary to the region surrounding the translation start site of CARD-3 mRNA, e.g., an oligonucleotide having the sequence CCCTGGTACTTGCCCCCTCCGGTAG (SEQ ID NO:34) or CCTGGTACTTGCCCCCTCC (SEQ ID NO:35) or of the CARD-4L mRNA

15 e.g., TCGTTAAGCCCTTGAAGACAGTG (SEQ ID NO:36) and TCGTTAGCCCTTGAAGACCAGTGAGTGTAG (SEQ ID NO:37). An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can

20 be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously

25 modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used.

30 Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil,

35 5-carboxymethylaminomethyl-2-thiouridine,

- 38 -

5-carboxymethylaminomethyluracil, dihydrouracil,  
beta-D-galactosylqueosine, inosine,  
N6-isopentenyladenine, 1-methylguanine, 1-methylinosine,  
2,2-dimethylguanine, 2-methyladenine, 2-methylguanine,  
5 3-methylcytosine, 5-methylcytosine, N6-adenine,  
7-methylguanine, 5-methylaminomethyluracil,  
5-methoxyaminomethyl-2-thiouracil,  
beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil,  
5-methoxyuracil, 2-methylthio-N6-isopentenyladenine,  
10 uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil,  
queosine, 2-thiocytosine, 5-methyl-2-thiouracil,  
2-thiouracil, 4-thiouracil, 5-methyluracil,  
uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic  
acid (v), 5-methyl-2-thiouracil,  
15 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and  
2,6-diaminopurine. Alternatively, the antisense nucleic  
acid can be produced biologically using an expression  
vector into which a nucleic acid has been subcloned in an  
antisense orientation (i.e., RNA transcribed from the  
20 inserted nucleic acid will be of an antisense orientation  
to a target nucleic acid of interest, described further  
in the following subsection).

The antisense nucleic acid molecules of the  
invention are typically administered to a subject or  
25 generated in situ such that they hybridize with or bind  
to cellular mRNA and/or genomic DNA encoding a CARD-3 or  
CARD-4 protein to thereby inhibit expression of the  
protein, e.g., by inhibiting transcription and/or  
translation. The hybridization can be by conventional  
30 nucleotide complementarity to form a stable duplex, or,  
for example, in the case of an antisense nucleic acid  
molecule which binds to DNA duplexes, through specific  
interactions in the major groove of the double helix. An  
example of a route of administration of antisense nucleic  
35 acid molecules of the invention include direct injection

- 39 -

at a tissue site. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be  
5 modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, e.g., by linking the antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also  
10 be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are  
15 preferred.

An antisense nucleic acid molecule of the invention can be an  $\alpha$ -anomeric nucleic acid molecule. An  $\alpha$ -anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which,  
20 contrary to the usual  $\beta$ -units, the strands run parallel to each other (Gaultier et al. (1987) Nucleic Acids. Res. 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-O-methylribonucleotide (Inoue et al. (1987) Nucleic Acids Res. 15:6131-6148) or a chimeric  
25 RNA-DNA analogue (Inoue et al. (1987) FEBS Lett. 215:327-330).

The invention also encompasses ribozymes. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded  
30 nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (e.g., hammerhead ribozymes (described in Haselhoff and Gerlach (1988) Nature 334:585-591)) can be used to catalytically cleave CARD-3 or CARD-4 mRNA transcripts to thereby inhibit  
35 translation of CARD-3 or CARD-4 mRNA. A ribozyme having

- 40 -

specificity for a CARD-3 or CARD-4-encoding nucleic acid can be designed based upon the nucleotide sequence of a CARD-3 or CARD-4 cDNA disclosed herein (e.g., SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:38, SEQ ID NO:40, and SEQ ID NO:42). For example, a derivative of a Tetrahymena L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in a CARD-3 or CARD-4-encoding mRNA. See, e.g., Cech et al. U.S. Patent No. 4,987,071; and Cech et al. U.S. Patent No. 5,116,742. Alternatively, CARD-3 or CARD-4 mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, e.g., Bartel and Szostak (1993) Science 261:1411-1418.

The invention also encompasses nucleic acid molecules which form triple helical structures. For example, CARD-3 or CARD-4 gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the CARD-3 or CARD-4 (e.g., the CARD-3 or CARD-4 promoter and/or enhancers) to form triple helical structures that prevent transcription of the CARD-3 or CARD-4 gene in target cells. See generally, Helene (1991) Anticancer Drug Des. 6(6):569-84; Helene (1992) Ann. N.Y. Acad. Sci. 660:27-36; and Maher (1992) Bioassays 14(12):807-15.

In embodiments, the nucleic acid molecules of the invention can be modified at the base moiety, sugar moiety or phosphate backbone to improve, e.g., the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acids can be modified to generate peptide nucleic acids (see Hyrup et al. (1996) Bioorganic & Medicinal Chemistry 4(1): 5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid



- 41 -

mimics, e.g., DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to  
5 allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup et al. (1996) supra; Perry-O'Keefe et al. (1996) Proc. Natl.  
10 Acad. Sci. USA 93: 14670-675.

PNAs of CARD-3 or CARD-4 can be used for therapeutic and diagnostic applications. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, e.g.,  
15 inducing transcription or translation arrest or inhibiting replication. PNAs of CARD-3 or CARD-4 can also be used, e.g., in the analysis of single base pair mutations in a gene by, e.g., PNA directed PCR clamping; as 'artificial restriction enzymes when used in  
20 combination with other enzymes, e.g., S1 nucleases (Hyrup (1996) supra; or as probes or primers for DNA sequence and hybridization (Hyrup (1996) supra; Perry-O'Keefe et al. (1996) Proc. Natl. Acad. Sci. USA 93: 14670-675).

In another embodiment, PNAs of CARD-3 or CARD-4  
25 can be modified, e.g., to enhance their stability or cellular uptake, by attaching lipophilic or other helper groups to PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques of drug delivery known in the art. For example, PNA-DNA chimeras  
30 of CARD-3 or CARD-4 can be generated which may combine the advantageous properties of PNA and DNA. Such chimeras allow DNA recognition enzymes, e.g., RNase H and DNA polymerases, to interact with the DNA portion while the PNA portion would provide high binding affinity and  
35 specificity. PNA-DNA chimeras can be linked using

- 42 -

linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup (1996) *supra*). The synthesis of PNA-DNA chimeras can be performed as described in Hyrup  
5 (1996) *supra* and Finn et al. (1996) *Nucleic Acids Research* 24(17):3357-63. For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry and modified nucleoside analogs, e.g.,  
10 5'-(4-methoxytrityl)amino-5'-deoxy-thymidine phosphoramidite, can be used as a between the PNA and the 5' end of DNA (Mag et al. (1989) *Nucleic Acid Res.* 17:5973-88). PNA monomers are then coupled in a stepwise manner to produce a chimeric molecule with a 5' PNA  
15 segment and a 3' DNA segment (Finn et al. (1996) *Nucleic Acids Research* 24(17):3357-63). Alternatively, chimeric molecules can be synthesized with a 5' DNA segment and a 3' PNA segment (Peterser et al. (1975) *Bioorganic Med. Chem. Lett.* 5:1119-11124).

20 In other embodiments, the oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, e.g., Letsinger et al. (1989) *Proc. Natl. Acad. Sci. USA*  
25 86:6553-6556; Lemaitre et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:648-652; PCT Publication No. W0 88/09810) or the blood-brain barrier (see, e.g., PCT Publication No. W0 89/10134). In addition, oligonucleotides can be modified with hybridization-triggered cleavage agents  
30 (see, e.g., Krol et al. (1988) *Bio/Techniques* 6:958-976) or intercalating agents (see, e.g., Zon (1988) *Pharm. Res.* 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport  
35 agent, hybridization-triggered cleavage agent, etc.

- 43 -

## II. Isolated CARD-3 or CARD-4 Proteins and Anti-CARD-3 or CARD-4 Antibodies.

One aspect of the invention pertains to isolated CARD-3 or CARD-4 proteins, and biologically active portions thereof, as well as polypeptide fragments suitable for use as immunogens to raise anti-CARD-3 or CARD-4 antibodies. In one embodiment, native CARD-3 or CARD-4 proteins can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. In another embodiment, CARD-3 or CARD-4 proteins are produced by recombinant DNA techniques. Alternative to recombinant expression, a CARD-3 or CARD-4 protein or polypeptide can be synthesized chemically using standard peptide synthesis techniques.

An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the CARD-3 or CARD-4 protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of CARD-3 or CARD-4 protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. Thus, CARD-3 or CARD-4 protein that is substantially free of cellular material includes preparations of CARD-3 or CARD-4 protein having less than about 30%, 20%, 10%, or 5% (by dry weight) of non-CARD-3 or CARD-4 protein (also referred to herein as a "contaminating protein"). When the CARD-3 or CARD-4 protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20%, 10%, or 5%

- 44 -

of the volume of the protein preparation. When CARD-3 or CARD-4 protein is produced by chemical synthesis, it is preferably substantially free of chemical precursors or other chemicals, i.e., it is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. Accordingly such preparations of CARD-3 or CARD-4 protein have less than about 30%, 20%, 10%, 5% (by dry weight) of chemical precursors or non-CARD-3 or CARD-4 chemicals.

Biologically active portions of a CARD-3 or CARD-4 protein include peptides comprising amino acid sequences sufficiently identical to or derived from the amino acid sequence of the CARD-3 or CARD-4 protein (e.g., the amino acid sequence shown in SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:26, SEQ ID NO:39, SEQ ID NO:41 or SEQ ID NO:43), which include less amino acids than the full length CARD-3 or CARD-4 proteins, and exhibit at least one activity of a CARD-3 or CARD-4 protein. Typically, biologically active portions comprise a domain or motif with at least one activity of the CARD-3 or CARD-4 protein. A biologically active portion of a CARD-3 or CARD-4 protein can be a polypeptide which is, for example, 10, 25, 50, 100 or more amino acids in length. Preferred biologically active polypeptides include one or more identified CARD-3 or CARD-4 structural domains, e.g., the CARD domain (SEQ ID NO:6 or SEQ ID NO:10 or SEQ ID NO:27).

Moreover, other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the functional activities of a native CARD-3 or CARD-4 protein.

CARD-3 or CARD-4 protein has the amino acid sequence shown of SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:26, SEQ ID NO:39, SEQ ID NO:41 or SEQ ID NO:43. Other useful CARD-3 or CARD-4 proteins are substantially identical to

- 45 -

SEQ ID NO:2 or SEQ ID NO:8 or SEQ ID NO:26, SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID NO:43 and retain the functional activity of the protein of SEQ ID NO:2 or SEQ ID NO:8 or SEQ ID NO:26, SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID NO:43 yet differ in amino acid sequence due to natural allelic variation or mutagenesis. CARD-3 and CARD-4 are involved in activating caspases in the apoptotic pathway. For example, in Example 10, CARD-4 is shown to enhance caspase 9 activity. Accordingly, a useful CARD-3 or CARD-4 protein is a protein which includes an amino acid sequence at least about 45%, preferably 55%, 65%, 75%, 85%, 95%, or 99% identical to the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:8 or SEQ ID NO:26, SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID NO:43 and retains the functional activity of the CARD-3 or CARD-4 proteins of SEQ ID NO:2 or SEQ ID NO:8 or SEQ ID NO:26, SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID NO:43. In other instances, the CARD-3 or CARD-4 protein is a protein having an amino acid sequence 55%, 65%, 75%, 85%, 95%, or 98% identical to the CARD-3 or CARD-4L CARD domain (SEQ ID NO:6, SEQ ID NO:10 and SEQ ID NO:27). In an embodiment, the CARD-3 or CARD-4 protein retains a functional activity of the CARD-3 or CARD-4 protein of SEQ ID NO:2, SEQ ID NO:8 or SEQ ID NO:26, SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID NO:43.

To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in

- 46 -

the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = #  
5 of identical positions/total # of positions x 100).

The determination of percent homology between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two  
10 sequences is the algorithm of Karlin and Altschul (1990) Proc. Nat'l Acad. Sci. USA 87:2264-2268, modified as in Karlin and Altschul (1993) Proc. Nat'l Acad. Sci. USA 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al. (1990) J.  
15 Mol. Biol. 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences similar or homologous to CARD-3 or CARD-4 nucleic acid molecules of the invention. For example, Example 5 describes the use  
20 of the TBLASTN program to query a database of sequences of full length and partial cDNA sequences with the human CARD-4 polypeptide sequence leading to the discovery of murine CARD-4 and Example 4 describes the use of BLASTN to query a proprietary EST database with the 5'  
25 untranslated sequence of CARD-4 leading to the discovery of two human CARD-4 splice variants. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to CARD-3 or CARD-4 protein molecules of the  
30 invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g.,  
35 XBLAST and NBLAST) can be used. See

- 47 -

<http://www.ncbi.nlm.nih.gov>. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

10 The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, only exact matches are counted.

15 chimeric or fusion proteins. As used herein, a CARD-4 CARD-4 "chimeric protein" or "fusion protein" comprises a CARD-3 or CARD-4 polypeptide operatively linked to a non-CARD-3 or CARD-4 polypeptide. A "CARD-3 or CARD-4 polypeptide" refers to a polypeptide having an amino acid sequence corresponding to CARD-3 or CARD-4L/S, murine CARD-4 or human CARD-4 splice variants, whereas a "non-CARD-3 or CARD-4 polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially identical to the CARD-3 or CARD-4L/S protein, murine CARD-4, or human CARD-4 splice variants e.g., a protein which is different from the CARD-3 or CARD-4 proteins and which is derived or CARD-4L fusion protein, the CARD-3 or CARD-4 polypeptide can correspond to all or a portion of a biologically active portion of a CARD-3 or CARD-4 protein. Within the fusion protein, the term "operatively linked" is intended to indicate that the

20  
25  
30  
35

CARD-3 or CARD-4 polypeptide and the non-CARD-3 or

- 48 -

non-CARD-4 polypeptide are fused in-frame to each other. The non-CARD-3 or non-CARD-4 polypeptide can be fused to the N-terminus or C-terminus of the CARD-3 or CARD-4 polypeptide.

5           One useful fusion protein is a GST-CARD-3 or GST-CARD-4 fusion protein in which the CARD-3 or CARD-4 sequences are fused to the C-terminus of the GST sequences. Such fusion proteins can facilitate the purification of recombinant CARD-3 or CARD-4.

10           In another embodiment, the fusion protein contains a signal sequence from another protein. In certain host cells (e.g., mammalian host cells), expression and/or secretion of CARD-3 or CARD-4 can be increased through use of a heterologous signal sequence. For example, the  
15 gp67 secretory sequence of the baculovirus envelope protein can be used as a heterologous signal sequence (Current Protocols in Molecular Biology, Ausubel et al., eds., John Wiley & Sons, 1992). Other examples of eukaryotic heterologous signal sequences include the  
20 secretory sequences of melittin and human placental alkaline phosphatase (Stratagene; La Jolla, California). In yet another example, useful prokaryotic heterologous signal sequences include the phoA secretory signal (Molecular cloning, Sambrook et al, second edition, Cold  
25 spring harbor laboratory press, 1989) and the protein A secretory signal (Pharmacia Biotech; Piscataway, New Jersey).

          In yet another embodiment, the fusion protein is a CARD-3 or CARD-4-immunoglobulin fusion protein in which  
30 all or part of CARD-3 or CARD-4 is fused to sequences derived from a member of the immunoglobulin protein family. The CARD-3 or CARD-4-immunoglobulin fusion proteins of the invention can be incorporated into pharmaceutical compositions and administered to a subject  
35 to inhibit an interaction between a CARD-3 or CARD-4



- 49 -

ligand and a CARD-3 or CARD-4 protein on the surface of a cell, to thereby suppress CARD-3 or CARD-4-mediated signal transduction in vivo. The CARD-3 or CARD-4-immunoglobulin fusion proteins can be used to  
5 affect the bioavailability of a CARD-3 or CARD-4 cognate ligand. Inhibition of the CARD-3 ligand/CARD-3 or CARD-4 ligand/CARD-4 interaction may be useful therapeutically for both the treatment of proliferative and  
10 differentiative disorders, as well as modulating (e.g. promoting or inhibiting) cell survival. Moreover, the CARD-3 or CARD-4-immunoglobulin fusion proteins of the invention can be used as immunogens to produce anti-CARD-3 or CARD-4 antibodies in a subject, to purify CARD-3 or CARD-4 ligands and in screening assays to  
15 identify molecules which inhibit the interaction of CARD-3 or CARD-4 with a CARD-3 or CARD-4 ligand.

Preferably, a CARD-3 or CARD-4 chimeric or fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments  
20 coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in  
25 of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR  
30 amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, e.g., Current Protocols in  
35 Molecular Biology, Ausubel et al. eds., John Wiley &

- 50 -

Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (e.g., a GST polypeptide). A CARD-3 or CARD-4-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the CARD-3 or CARD-4 protein.

The present invention also pertains to variants of the CARD-3 or CARD-4 proteins which function as either CARD-3 or CARD-4 agonists (mimetics) or as CARD-3 or CARD-4 antagonists. Variants of the CARD-3 or CARD-4 protein can be generated by mutagenesis, e.g., discrete point mutation or truncation of the CARD-3 or CARD-4 protein. An agonist of the CARD-3 or CARD-4 protein can retain substantially the same, or a subset, of the biological activities of the naturally occurring form of the CARD-3 or CARD-4 protein. An antagonist of the CARD-3 or CARD-4 protein can inhibit one or more of the activities of the naturally occurring form of the CARD-3 or CARD-4 protein by, for example, competitively binding to a downstream or upstream member of a cellular signaling cascade which includes the CARD-3 or CARD-4 protein. Thus, specific biological effects can be elicited by treatment with a variant of limited function. Treatment of a subject with a variant having a subset of the biological activities of the naturally occurring form of the protein can have fewer side effects in a subject relative to treatment with the naturally occurring form of the CARD-3 or CARD-4 proteins.

Variants of the CARD-3 or CARD-4 protein which function as either CARD-3 or CARD-4 agonists (mimetics) or as CARD-3 or CARD-4 antagonists can be identified by screening combinatorial libraries of mutants, e.g., truncation mutants of the CARD-3 or CARD-4 protein for CARD-3 or CARD-4 protein agonist or antagonist activity. In one embodiment, a variegated library of CARD-3 or

- 51 -

CARD-4 variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of CARD-3 or CARD-4 variants can be produced by, for example, enzymatically  
5 ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential CARD-3 or CARD-4 sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display) containing the set of  
10 CARD-3 or CARD-4 sequences therein. There are a variety of methods which can be used to produce libraries of potential CARD-3 or CARD-4 variants from a degenerate oligonucleotide sequence. Chemical synthesis of a  
15 degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential CARD-3 or CARD-4 sequences. Methods for  
20 synthesizing degenerate oligonucleotides are known in the art (see, e.g., Narang (1983) Tetrahedron 39:3; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477).

25        Useful fragments of CARD-3 and CARD-4 include fragments comprising or consisting of a domain or subdomain described herein, e.g., a kinase domain or a CARD domain.

In addition, libraries of fragments of the CARD-3  
30 or CARD-4 protein coding sequence can be used to generate a variegated population of CARD-3 or CARD-4 fragments for screening and subsequent selection of variants of a CARD-3 or CARD-4 protein. In one embodiment, a library of coding sequence fragments can be generated by treating  
35 a double stranded PCR fragment of a CARD-3 or CARD-4

- 52 -

coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense  
5 pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an  
10 N-terminal and internal fragments of various sizes of the CARD-3 or CARD-4 protein.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA  
15 libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of CARD-3 or CARD-4 proteins. The most widely used  
20 techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the  
25 combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to  
30 identify CARD-3 or CARD-4 variants (Arkin and Yourvan (1992) Proc. Natl. Acad. Sci. USA 89:7811-7815; Delgrave et al. (1993) Protein Engineering 6(3):327-331).

An isolated CARD-3 or CARD-4 protein, or a portion or fragment thereof, can be used as an immunogen to  
35 generate antibodies that bind CARD-3 or CARD-4 using

- 53 -

standard techniques for polyclonal and monoclonal antibody preparation. The full-length CARD-3 or CARD-4 protein can be used or, alternatively, the invention provides antigenic peptide fragments of CARD-3 or CARD-4 for use as immunogens. The antigenic peptide of CARD-3 or CARD-4 comprises at least 8 (preferably 10, 15, 20, or 30) amino acid residues of the amino acid sequence shown in SEQ ID NO:2, SEQ ID NO:8 or SEQ ID NO:26, or SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID NO:43 or polypeptides including amino acids 128-139 or 287-298 of human CARD-4L and encompasses an epitope of CARD-3 or CARD-4 such that an antibody raised against the peptide forms a specific immune complex with CARD-3 or CARD-4.

Useful antibodies include antibodies which bind to a domain or subdomain of CARD-3 or CARD-4 described herein, e.g., a kinase domain or a CARD domain).

Preferred epitopes encompassed by the antigenic peptide are regions of CARD-3 or CARD-4 that are located on the surface of the protein, e.g., hydrophilic regions. Other important criteria include a preference for a terminal sequence, high antigenic index (e.g., as predicted by Jameson-Wolf algorithm), ease of peptide synthesis (e.g., avoidance of prolines); and high surface probability (e.g., as predicted by the Emini algorithm; Figure 8 and Figure 9).

A CARD-3 or CARD-4 immunogen typically is used to prepare antibodies by immunizing a suitable subject, (e.g., rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, recombinantly expressed CARD-3 or CARD-4 protein or a chemically synthesized CARD-3 or CARD-4 polypeptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic

- 54 -

CARD-3 or CARD-4 preparation induces a polyclonal anti-CARD-3 or CARD-4 antibody response. For example, polypeptides including amino acids 128-139 or 287-298 of human CARD-4L were conjugated to KLH and the resulting  
5 conjugates were used to immunize rabbits and polyclonal antibodies that specifically recognize the two immunogen peptides were generated.

Accordingly, another aspect of the invention pertains to anti-CARD-3 or CARD-4 antibodies. The term  
10 "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically binds an antigen, such as CARD-3 or CARD-4. A molecule which specifically  
15 binds to CARD-3 or CARD-4 is a molecule which binds CARD-3 or CARD-4, but does not substantially bind other molecules in a sample, e.g., a biological sample, which naturally contains CARD-3 or CARD-4. Examples of immunologically active portions of immunoglobulin  
20 molecules include F(ab) and F(ab')<sub>2</sub> fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies that bind CARD-3 or CARD-4. The term "monoclonal antibody" or "monoclonal antibody  
25 composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of CARD-3 or CARD-4. A monoclonal antibody composition thus typically displays a single  
30 binding affinity for a particular CARD-3 or CARD-4 protein with which it immunoreacts.

Polyclonal anti-CARD-3 or CARD-4 antibodies can be prepared as described above by immunizing a suitable subject with a CARD-3 or CARD-4 immunogen. The  
35 anti-CARD-3 or CARD-4 antibody titer in the immunized

- 55 -

subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized CARD-3 or CARD-4. If desired, the antibody molecules directed against CARD-3  
5 or CARD-4 can be isolated from the mammal (e.g., from the blood) and further purified by well-known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, e.g., when the anti-CARD-3 or CARD-4 antibody titers are  
10 highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497, the human B cell hybridoma technique (Kozbor  
15 et al. (1983) *Immunol Today* 4:72), the EBV-hybridoma technique (Cole et al. (1985), *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing various antibodies monoclonal antibody hybridomas is well known  
20 (see generally *Current Protocols in Immunology* (1994) Coligan et al. (eds.) John Wiley & Sons, Inc., New York, NY). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a CARD-3 or CARD-4 immunogen  
25 as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds CARD-3 or CARD-4.

Any of the many well known protocols used for  
30 fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-CARD-3 or CARD-4 monoclonal antibody (see, e.g., *Current Protocols in Immunology*, supra; Galfre et al. (1977) *Nature* 266:55052; R.H. Kenneth, in *Monoclonal Antibodies: A New*  
35 *Dimension In Biological Analyses*, Plenum Publishing

- 56 -

Corp., New York, New York (1980); and Lerner (1981) Yale J. Biol. Med., 54:387-402. Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods which also would be useful.

5 Typically, the immortal cell line (e.g., a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an

10 immortalized mouse cell line, e.g., a myeloma cell line that is sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, e.g.,

15 the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from ATCC. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then

20 selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture

25 supernatants for antibodies that bind CARD-3 or CARD-4, e.g., using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-CARD-3 or CARD-4 antibody can be identified and isolated by

30 screening a recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library) with CARD-3 or CARD-4 to thereby isolate immunoglobulin library members that bind CARD-3 or CARD-4. Kits for generating and screening phage display libraries are

35 commercially available (e.g., the Pharmacia Recombinant



- 57 -

Phage Antibody System, Catalog No. 27-9400-01; and the Stratagene SurfZAP Phage Display Kit, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening  
5 antibody display library can be found in, for example, U.S. Patent No. 5,223,409; PCT Publication No. WO 92/18619; PCT Publication No. WO 91/17271; PCT Publication No. WO 92/20791; PCT Publication No. WO 92/15679; PCT Publication No. WO 93/01288; PCT  
10 Publication No. WO 92/01047; PCT Publication No. WO 92/09690; PCT Publication No. WO 90/02809; Fuchs et al. (1991) Bio/Technology 9:1370-1372; Hay et al. (1992) Hum. Antibod. Hybridomas 3:81-85; Huse et al. (1989) Science 246:1275-1281; Griffiths et al. (1993) EMBO J 12:725-734.  
15        Additionally, recombinant anti-CARD-3 or CARD-4 antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such  
20 chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in PCT Publication No. WO 87/02671; European Patent Application 184,187; European Patent Application 171,496; European Patent  
25 Application 173,494; PCT Publication No. WO 86/01533; U.S. Patent No. 4,816,567; European Patent Application 125,023; Better et al. (1988) Science 240:1041-1043; Liu et al. (1987) Proc. Natl. Acad. Sci. USA 84:3439-3443; Liu et al. (1987) J. Immunol. 139:3521-3526; Sun et al.  
30 (1987) Proc. Natl. Acad. Sci. USA 84:214-218; Nishimura et al. (1987) Canc. Res. 47:999-1005; Wood et al. (1985) Nature 314:446-449; and Shaw et al. (1988) J. Natl. Cancer Inst. 80:1553-1559; Morrison, (1985) Science 229:1202-1207; Oi et al. (1986) Bio/Techniques 4:214;  
35 U.S. Patent 5,225,539; Jones et al. (1986) Nature

- 58 -

321:552-525; Verhoeyan et al. (1988) Science 239:1534; and Beidler et al. (1988) J. Immunol. 141:4053-4060.

An anti-CARD-3 or CARD-4 antibody (e.g., monoclonal antibody) can be used to isolate CARD-3 or  
5 CARD-4 by standard techniques, such as affinity chromatography or immunoprecipitation. An anti-CARD-3 or CARD-4 antibody can facilitate the purification of natural CARD-3 or CARD-4 from cells and of recombinantly produced CARD-3 or CARD-4 expressed in host cells.  
10 Moreover, an anti-CARD-3 or CARD-4 antibody can be used to detect CARD-3 or CARD-4 protein (e.g., in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the CARD-3 or CARD-4 protein. Anti-CARD-3 or CARD-4 antibodies can be  
15 used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, e.g., to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of  
20 detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase,  $\beta$ -galactosidase, or  
25 acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine  
30 fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$  or  $^3\text{H}$ .

- 59 -

### III. Recombinant Expression Vectors and Host Cells

Another aspect of the invention pertains to vectors, preferably expression vectors, containing a nucleic acid encoding CARD-3 or CARD-4 (or a portion thereof). As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors, expression vectors, are capable of directing the expression of genes to which they are operatively linked. In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids (vectors). However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic

- 60 -

acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for  
5 expression of the nucleotide sequence (e.g., in an in vitro transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to include promoters, enhancers and other expression control  
10 elements (e.g., polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct  
15 constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design  
20 of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including  
25 fusion proteins or peptides, encoded by nucleic acids as described herein (e.g., CARD-3 or CARD-4 proteins, mutant forms of CARD-3 or CARD-4, fusion proteins, etc.).

The recombinant expression vectors of the invention can be designed for expression of CARD-3 or  
30 CARD-4 in prokaryotic or eukaryotic cells, e.g., bacterial cells such as E. coli, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, Gene Expression Technology: Methods in  
35 Enzymology 185, Academic Press, San Diego, CA (1990).

- 61 -

Alternatively, the recombinant expression vector can be transcribed and translated in vitro, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith and Johnson (1988) Gene 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann et al., (1988) Gene 69:301-315) and pET 11d (Studier et al., Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, California (1990) 60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac

- 62 -

fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 *gn10*-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 *gn1*). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident ? prophage harboring a T7 *gn1* gene under the transcriptional control of the lacUV5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada et al. (1992) Nucleic Acids Res. 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the CARD-3 or CARD-4 expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari et al. (1987) EMBO J. 6:229-234), pMFa (Kurjan and Herskowitz, (1982) Cell 30:933-943), pJRY88 (Schultz et al. (1987) Gene 54:113-123), pYES2 (Invitrogen Corporation, San Diego, CA), pGBT9 (Clontech, Palo Alto, CA), pGAD10 (Clontech, Palo Alto, CA), pYADE4 and pYGAE2 and pYPGE2 (Brunelli and Pall, (1993) Yeast 9:1299-1308), pYPGE15 (Brunelli and Pall, (1993) Yeast 9:1309-1318), pACTII (Dr. S.E. Elledge, Baylor College of Medicine), and picZ (Invitrogen Corp, San Diego, CA). For example, in Example 7 the expression of a fusion protein comprising amino acids 1-145 of human CARD-4L fused to the DNA-binding domain of *S. cerevisiae*

- 63 -

transcription factor GAL4 from the yeast expression vector pGBT9 is described. In another example, Example 8 describes the expression of a fusion protein comprising amino acids 406-953 of human CARD-4L fused to the

5 DNA-binding domain of *S. cerevisiae* transcription factor GAL4 from the yeast expression vector pGBT9. In yet another example, Example 7 describes the expression of a fusion protein comprising CARD-3 fused to the transcriptional activation domain of *S. cerevisiae*

10 transcription factor GAL4 from the yeast expression vector pACTII.

Alternatively, CARD-3 or CARD-4 can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins

15 in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith et al. (1983) Mol. Cell Biol. 3:2156-2165) and the pVL series (Lucklow and Summers (1989) Virology 170:31-39).

In yet another embodiment, a nucleic acid of the

20 invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed (1987) Nature 329:840), pCI (Promega), and pMT2PC (Kaufman et al. (1987) EMBO J. 6:187-195). When used in mammalian cells,

25 the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic

30 and eukaryotic cells see chapters 16 and 17 of Sambrook et al. (supra). For example, Example 9, Example 10, and Example 12 describe the expression of human CARD-4 or fragments thereof, CARD-3, or both from the mammalian expression vector pCI.

- 64 -

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert et al. (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji et al. (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund et al. (1985) *Science* 230:912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the  $\alpha$ -fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to CARD-3 or CARD-4 mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the



- 65 -

antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense  
5 RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into  
10 which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub et al. (Reviews - Trends in Genetics, Vol. 1(1) 1986).

Another aspect of the invention pertains to host  
15 cells into which a recombinant expression vector of the invention or isolated nucleic acid molecule of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the  
20 particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are  
25 still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, CARD-3 or CARD-4 protein can be expressed in bacterial cells such as *E. coli*, insect  
30 cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art. For example, in Example 7 a *Saccharomyces cerevisiae* host cell for recombinant CARD-4 and CARD-3 expression is  
35 described and in Examples 9, 10, and 12 293T host cells

- 66 -

for expression of CARD4 or fragments thereof or CARD-3 are described.

Vector DNA or an isolated nucleic acid molecule of the invention can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, et al. (supra), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. IN some cases vector DNA is retained by the host cell. In other cases the host cell does not retain vector DNA and retains only an isolated nucleic acid molecule of the invention carried by the vector. In some cases, and isolated nucleic acid molecule of the invention is used to transform a cell without the use of a vector.

In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding CARD-3 or CARD-4 or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug

- 67 -

selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a  
5 prokaryotic or eukaryotic host cell in culture, can be used to produce a (i.e., express) CARD-3 or CARD-4 protein. Accordingly, the invention further provides methods for producing CARD-3 or CARD-4 protein using the host cells of the invention. In one embodiment, the  
10 method comprises culturing the host cell of the invention (into which a recombinant expression vector or isolated nucleic acid molecule encoding CARD-3 or CARD-4 has been introduced) in a suitable medium such that CARD-3 or CARD-4 protein is produced. In another embodiment, the  
15 method further comprises isolating CARD-3 or CARD-4 from the medium or the host cell.

The host cells of the invention can also be used to produce nonhuman transgenic animals. For example, in one embodiment, a host cell of the invention is a  
20 fertilized oocyte or an embryonic stem cell into which CARD-3 or CARD-4-coding sequences have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous CARD-3 or CARD-4 sequences have been introduced into their genome or  
25 homologous recombinant animals in which endogenous CARD-3 or CARD-4 sequences have been altered. Such animals are useful for studying the function and/or activity of CARD-3 or CARD-4 and for identifying and/or evaluating modulators of CARD-3 or CARD-4 activity. As used herein,  
30 a "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats,  
35 chickens, amphibians, etc. A transgene is exogenous DNA

- 68 -

which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, an "homologous recombinant animal" is a non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous CARD-3 or CARD-4 gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, e.g., an embryonic cell of the animal, prior to development of the animal.

A transgenic animal of the invention can be created by introducing CARD-3 or CARD-4-encoding nucleic acid into the male pronuclei of a fertilized oocyte, e.g., by microinjection, retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. The CARD-3 or CARD-4 cDNA sequence e.g., that of (SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42 or the cDNA of ATCC \_\_\_\_\_, or the cDNA of ATCC \_\_\_\_\_, or the cDNA of ATCC \_\_\_\_\_) can be introduced as a transgene into the genome of a non-human animal. Alternatively, a nonhuman homolog or ortholog of the human CARD-3 or CARD-4 gene, such as a mouse CARD-3 or CARD-4 gene, can be isolated based on hybridization to the human CARD-3 or CARD-4 cDNA and used as a transgene. For example, the mouse ortholog of CARD-4, Figure 15 and SEQ ID NO:42 can be used to make a transgenic animal using standard methods. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to the CARD-3 or CARD-4 transgene to direct expression of CARD-3 or CARD-4 protein to

- 69 -

particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, U.S. Patent No. 4,873,191 and in Hogan, Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of the CARD-3 or CARD-4 transgene in its genome and/or expression of CARD-3 or CARD-4 mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding CARD-3 or CARD-4 can further be bred to other transgenic animals carrying other transgenes.

To create an homologous recombinant animal, a vector is prepared which contains at least a portion of a CARD-3 or CARD-4 gene (e.g., a human or a non-human homolog of the CARD-3 or CARD-4 gene, e.g., a murine CARD-3 or CARD-4 gene) into which a deletion, addition or substitution has been introduced to thereby alter, e.g., functionally disrupt, the CARD-3 or CARD-4 gene. In an embodiment, the vector is designed such that, upon homologous recombination, the endogenous CARD-3 or CARD-4 gene is functionally disrupted (i.e., no longer encodes a functional protein; also referred to as a "knock out" vector). Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous CARD-3 or CARD-4 gene is mutated or otherwise altered but still encodes functional protein (e.g., the upstream regulatory region can be altered to thereby alter the expression of the endogenous CARD-3 or CARD-4 protein). In the homologous recombination vector, the altered

- 70 -

portion of the CARD-3 or CARD-4 gene is flanked at its 5' and 3' ends by additional nucleic acid of the CARD-3 or CARD-4 gene to allow for homologous recombination to occur between the exogenous CARD-3 or CARD-4 gene carried by the vector and an endogenous CARD-3 or CARD-4 gene in an embryonic stem cell. The additional flanking CARD-3 or CARD-4 nucleic acid is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see, e.g., Thomas and Capecchi (1987) Cell 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced CARD-3 or CARD-4 gene has homologously recombined with the endogenous CARD-3 or CARD-4 gene are selected (see, e.g., Li et al. (1992) Cell 69:915). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see, e.g., Bradley in Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination vectors and homologous recombinant animals are described further in Bradley (1991) Current Opinion in Bio/Technology 2:823-829 and in PCT Publication Nos. WO 90/11354, WO 91/01140, WO 92/0968, and WO 93/04169.

In another embodiment, transgenic non-humans animals can be produced which contain selected systems

- 71 -

which allow for regulated expression of the transgene. One example of such a system is the cre/loxP recombinase system of bacteriophage P1. For a description of the cre/loxP recombinase system, see, e.g., Lakso et al. (1992) Proc. Natl. Acad. Sci. USA 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman et al. (1991) Science 251:1351-1355. If a cre/loxP recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the Cre recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut et al. (1997) Nature 385:810-813 and PCT Publication Nos. WO 97/07668 and WO 97/07669. In brief, a cell, e.g., a somatic cell, from the transgenic animal can be isolated and induced to exit the growth cycle and enter Go phase. The quiescent cell can then be fused, e.g., through the use of electrical pulses, to an enucleated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyte and then transferred to pseudopregnant female foster animal. The offspring borne of this female foster animal will be a clone of the animal from which the cell, e.g., the somatic cell, is isolated.

- 72 -

#### IV. Pharmaceutical Compositions

The CARD-3 or CARD-4 nucleic acid molecules, CARD-3 or CARD-4 proteins, and anti-CARD-3 or CARD-4 antibodies (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as



- 73 -

acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral  
5 preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the  
10 extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL<sup>®</sup> (BASF; Parsippany, NJ) or phosphate buffered saline (PBS). In all cases,  
15 the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier  
20 can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a  
25 coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens,  
30 chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable

- 74 -

compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., a CARD-3 or CARD-4 protein or anti-CARD-3 or CARD-4 antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a

- 75 -

lubricant such as magnesium stearate or Sterotes; a  
glidant such as colloidal silicon dioxide; a sweetening  
agent such as sucrose or saccharin; or a flavoring agent  
such as peppermint, methyl salicylate, or orange  
5 flavoring. For administration by inhalation, the  
compounds are delivered in the form of an aerosol spray  
from pressured container or dispenser which contains a  
suitable propellant, e.g., a gas such as carbon dioxide,  
or a nebulizer.

10           Systemic administration can also be by  
transmucosal or transdermal means. For transmucosal or  
transdermal administration, penetrants appropriate to the  
barrier to be permeated are used in the formulation.  
Such penetrants are generally known in the art, and  
15 include, for example, for transmucosal administration,  
detergents, bile salts, and fusidic acid derivatives.  
Transmucosal administration can be accomplished through  
the use of nasal sprays or suppositories. For  
transdermal administration, the active compounds are  
20 formulated into ointments, salves, gels, or creams as  
generally known in the art.

The compounds can also be prepared in the form of  
suppositories (e.g., with conventional suppository bases  
such as cocoa butter and other glycerides) or retention  
25 enemas for rectal delivery.

In one embodiment, the active compounds are  
prepared with carriers that will protect the compound  
against rapid elimination from the body, such as a  
controlled release formulation, including implants and  
30 microencapsulated delivery systems. Biodegradable,  
biocompatible polymers can be used, such as ethylene  
vinyl acetate, polyanhydrides, polyglycolic acid,  
collagen, polyorthoesters, and polylactic acid. Methods  
for preparation of such formulations will be apparent to  
35 those skilled in the art. The materials can also be

- 76 -

obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as  
5 pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or  
10 parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active  
15 compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the  
20 particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors.  
25 Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (U.S. Patent 5,328,470) or by stereotactic injection (see, e.g., Chen et al. (1994) Proc. Natl. Acad. Sci. USA 91:3054-3057). The pharmaceutical preparation of the  
30 gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g.  
35 retroviral vectors, the pharmaceutical preparation can

- 77 -

include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with  
5 instructions for administration.

## V. Uses and Methods of the Invention

The nucleic acid molecules, proteins, protein homologues, and antibodies described herein can be used in one or more of the following methods: a) screening  
10 assays; b) detection assays (e.g., chromosomal mapping, tissue typing, forensic biology), c) predictive medicine (e.g., diagnostic assays, prognostic assays, monitoring clinical trials, and pharmacogenomics); and d) methods of treatment (e.g., therapeutic and prophylactic). A CARD-3  
15 or CARD-4 protein interacts with other cellular proteins and can thus be used for (i) regulation of cellular proliferation; (ii) regulation of cellular differentiation; and (iii) regulation of cell survival. The isolated nucleic acid molecules of the invention can  
20 be used to express CARD-3 or CARD-4 protein (e.g., via a recombinant expression vector in a host cell in gene therapy applications), to detect CARD-3 or CARD-4 mRNA (e.g., in a biological sample) or a genetic lesion in a CARD-3 or CARD-4 gene, and to modulate CARD-3 or CARD-4  
25 activity. In addition, the CARD-3 or CARD-4 proteins can be used to screen drugs or compounds which modulate the CARD-3 or CARD-4 activity or expression as well as to treat disorders characterized by insufficient or excessive production of CARD-3 or CARD-4 protein or  
30 production of CARD-3 or CARD-4 protein forms which have decreased or aberrant activity compared to CARD-3 or CARD-4 wild type protein. In addition, the anti-CARD-3 or CARD-4 antibodies of the invention can be used to

- 78 -

detect and isolate CARD-3 or CARD-4 proteins and modulate CARD-3 or CARD-4 activity.

This invention further pertains to novel agents identified by the above-described screening assays and  
5 uses thereof for treatments as described herein.

#### A. Screening Assays

The invention provides a method (also referred to herein as a "screening assay") for identifying modulators, i.e., candidate or test compounds or agents  
10 (e.g., peptides, peptidomimetics, small molecules or other drugs) which bind to CARD-3 or CARD-4 proteins or biologically active portions thereof or have a stimulatory or inhibitory effect on, for example, CARD-3 or CARD-4 expression or CARD-3 or CARD-4 activity. An  
15 example of a biologically active portion of human CARD-4 is amino acids 1-145 encoding the CARD domain which is sufficient to exhibit CARD-3-binding activity as described in Example 7. Amino acids 406-953 of human CARD4L comprising the LRR domain represent a biologically  
20 active portion of CARD-4L because they possess hNUDC-binding activity as described in Example 8.

In one embodiment, the invention provides assays for screening candidate or test compounds which bind to or modulate the activity of a CARD-3 or CARD-4 proteins  
25 or polypeptides or biologically active portions thereof. The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable  
30 parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide

- 79 -

libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam (1997) Anticancer Drug Des. 12:145). Examples of methods for the synthesis of  
5 molecular libraries can be found in the art, for example in: DeWitt et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90:6909; Erb et al. (1994) Proc. Natl. Acad. Sci. USA 91:11422; Zuckermann et al. (1994). J. Med. Chem. 37:2678; Cho et al. (1993) Science 261:1303; Carrell et  
10 al. (1994) Angew. Chem. Int. Ed. Engl. 33:2059; Carell et al. (1994) Angew. Chem. Int. Ed. Engl. 33:2061; and Gallop et al. (1994) J. Med. Chem. 37:1233.

Libraries of compounds may be presented in solution (e.g., Houghten (1992) Bio/Techniques  
15 13:412-421), or on beads (Lam (1991) Nature 354:82-84), chips (Fodor (1993) Nature 364:555-556), bacteria (U.S. Patent No. 5,223,409), spores (Patent Nos. 5,571,698; 5,403,484; and 5,223,409), plasmids (Cull et al. (1992) Proc. Natl. Acad. Sci. USA 89:1865-1869) or on phage  
20 (Scott and Smith (1990) Science 249:386-390; Devlin (1990) Science 249:404-406; Cwirla et al. (1990) Proc. Natl. Acad. Sci. 87:6378-6382; and Felici (1991) J. Mol. Biol. 222:301-310).

Determining the ability of the test compound to  
25 modulate the activity of CARD-3 or CARD-4 or a biologically active portion thereof can be accomplished, for example, by determining the ability of the CARD-3 or CARD-4 protein to bind to or interact with a CARD-3 or CARD-4 target molecule. As used herein, a "target  
30 molecule" is a molecule with which a CARD-3 or CARD-4 protein binds or interacts in nature, for example, a molecule associated with the internal surface of a cell membrane or a cytoplasmic molecule. A CARD-3 or CARD-4 target molecule can be a non-CARD-3 or CARD-4 molecule or  
35 a CARD-3 or CARD-4 protein or polypeptide of the present

- 80 -

invention. In one embodiment, a CARD-3 or CARD-4 target molecule is a component of an apoptotic signal transduction pathway, e.g., CARD-3 and CARD-4. The target, for example, can be a second intracellular  
5 protein which has catalytic activity or a protein which facilitates the association of downstream signaling molecules with CARD-3 or CARD-4. In another embodiment, CARD-3 or CARD-4 target molecules include CARD-3 because CARD-3 was found to bind to CARD-4 (Examples 7 and 12)  
10 and hNUDC because hNUDC was found to bind to CARD-4 (Example 8).

Determining the ability of the test compound to modulate the activity of CARD-3 or CARD-4 or a biologically active portion thereof can be accomplished,  
15 for example, by determining the ability of the CARD-3 or CARD-4 protein to bind to or interact with any of the specific proteins listed in the previous paragraph as CARD-3 or CARD-4 target molecules. In another embodiment, CARD-3 or CARD-4 target molecules include all  
20 proteins that bind to a CARD-3 or CARD-4 protein or fragment thereof in a two-hybrid system binding assay which can be used without undue experimentation to isolate such proteins from cDNA or genomic two-hybrid system libraries. For example, Example 7 describes the  
25 use of the CARD-4 CARD domain region to identify CARD-3 in a two-hybrid screen and Example 8 describes the use of the CARD-4 LRR region to identify hNUDC in a two-hybrid screen. The binding assays described in this section could be cell-based or cell free (described  
30 subsequently).

Determining the ability of the CARD-3 or CARD-4 protein to bind to or interact with a CARD-3 or CARD-4 target molecule can be accomplished by one of the methods described above for determining direct binding. In an  
35 embodiment, determining the ability of the CARD-3 or



- 81 -

CARD-4 protein to bind to or interact with a CARD-3 or CARD-4 target molecule can be accomplished by determining the activity of the target molecule. For example, the activity of the target molecule can be determined by

5 detecting induction of a cellular second messenger of the target (e.g., intracellular  $\text{Ca}^{2+}$ , diacylglycerol, IP<sub>3</sub>, etc.), detecting catalytic/enzymatic activity of the target on an appropriate substrate, detecting the induction of a reporter gene (e.g., a CARD-3 or

10 CARD-4-responsive regulatory element operatively linked to a nucleic acid encoding a detectable marker, e.g. luciferase), or detecting a cellular response, for example, cell survival, cellular differentiation, or cell proliferation. For example, in Example 12 CARD-4 is

15 shown to bind to CARD-3 and in Example 10, by monitoring a cellular response, CARD-4 is shown to enhance caspase 9 activity, cell death or apoptosis. Because CARD-3 and CARD-4 enhance caspase 9 activity, CARD-3 or CARD-4 activity can be monitored by assaying the caspase

20 9-mediated apoptosis cellular response or caspase 9 enzymatic activity. In addition, and in another embodiment, genes induced by CARD-3 or CARD-4 expression could be identified by expressing CARD-3 or CARD-4 in a cell line and conducting a transcriptional profiling

25 experiment wherein the mRNA expression patterns of the cell line transformed with an empty expression vector and the cell line transformed with a CARD-3 or CARD-4 expression vector are compared. The promoters of genes induced by CARD-3 or CARD-4 expression could be

30 operatively linked to reporter genes suitable for screening such as luciferase, secreted alkaline phosphatase, or beta-galactosidase and the resulting constructs could be introduced into appropriate expression vectors. A recombinant cell line containing

35 CARD-3 or CARD-4 and transfected with an expression

- 82 -

vector containing a CARD-3 or CARD-4 responsive promoter operatively linked to a reporter gene could be used to identify test compounds that modulate CARD-3 or CARD-4 activity by assaying the expression of the reporter gene in response to contacting the recombinant cell line with test compounds. CARD-3 or CARD-4 agonists can be identified as increasing the expression of the reporter gene and CARD-3 or CARD-4 antagonists can be identified as decreasing the expression of the reporter gene.

10 In another embodiment of the invention, the ability of a test compound to modulate the activity of CARD-3, CARD-4, or biologically active portions thereof can be determined by assaying the ability of the test compound to modulate CARD-3 or CARD-4-dependent pathways or processes where the CARD-3 or CARD-4 target proteins that mediate the CARD-3 or CARD-4 effect are known or unknown. Potential CARD-3 or CARD-4-dependent pathways or processes include but are not limited to the modulation of cellular signal transduction pathways and their related second messenger molecules (e.g., intracellular Ca<sup>2+</sup>, diacylglycerol, IP<sub>3</sub>, cAMP etc.), cellular enzymatic activities, cellular responses (e.g., cell survival, cellular differentiation, or cell proliferation), or the induction or repression of cellular or heterologous mRNAs or proteins. CARD-3 or CARD-4-dependent pathways or processes could be assayed by standard cell-based or cell free assays appropriate for the specific pathway or process under study. For example, Example 9 describes how expression of CARD-4S or CARD-4L in 293T cells induces the NF- $\kappa$ B pathway as determined by the measurement of a cotransfected NF- $\kappa$ B pathway luciferase reporter gene. In another embodiment, cells cotransfected with CARD-4 and the NF- $\kappa$ B luciferase reporter gene could be contacted with a test compound and test compounds that block CARD-4 activity could be

- 83 -

identified by their reduction of CARD-4-dependent NF- $\kappa$ B pathway luciferase reporter gene expression. Test compounds that agonize CARD-4 would be expected to increase reporter gene expression. In another  
5 embodiment, CARD-4 could be expressed in a cell line and the recombinant CARD-4-expressing cell line could be contacted with a test compound. Test compounds that inhibit CARD-4 activity could be indentified by their reduction of CARD-4-depended NF- $\kappa$ B pathway stimulation as  
10 measured by the assay of a NF- $\kappa$ B pathway reporter gene, NF- $\kappa$ B nuclear localization, I $\kappa$ B phosphorylation or proteolysis, or other standard assays for NF- $\kappa$ B pathway activation known to those skilled in the art.

In yet another embodiment, an assay of the present  
15 invention is a cell-free assay comprising contacting a CARD-3 or CARD-4 protein or biologically active portion thereof with a test compound and determining the ability of the test compound to bind to the CARD-3 or CARD-4 protein or biologically active portion thereof. Binding  
20 of the test compound to the CARD-3 or CARD-4 protein can be determined either directly or indirectly as described above. In one embodiment, a competitive binding assay includes contacting the CARD-3 or CARD-4 protein or biologically active portion thereof with a compound known  
25 to bind CARD-3 or CARD-4 to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a CARD-3 or CARD-4 protein, wherein determining the ability of the test compound to interact with a CARD-3 or  
30 CARD-4 protein comprises determining the ability of the test compound to preferentially bind to CARD-3 or CARD-4 or biologically active portion thereof as compared to the known binding compound.

In another embodiment, an assay is a cell-free  
35 assay comprising contacting CARD-3 or CARD-4 protein or

- 84 -

biologically active portion thereof with a test compound and determining the ability of the test compound to modulate (e.g., stimulate or inhibit) the activity of the CARD-3 or CARD-4 protein or biologically active portion thereof. Determining the ability of the test compound to modulate the activity of CARD-3 or CARD-4 can be accomplished, for example, by determining the ability of the CARD-3 or CARD-4 protein to bind to a CARD-3 or CARD-4 target molecule by one of the methods described above for determining direct binding. In an alternative embodiment, determining the ability of the test compound to modulate the activity of CARD-3 or CARD-4 can be accomplished by determining the ability of the CARD-3 or CARD-4 protein to further modulate a CARD-3 or CARD-4 target molecule. For example, the catalytic/enzymatic activity of the target molecule on an appropriate substrate can be determined as previously described.

In yet another embodiment, the cell-free assay comprises contacting the CARD-3 or CARD-4 protein or biologically active portion thereof with a known compound which binds CARD-3 or CARD-4 to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a CARD-3 or CARD-4 protein, wherein determining the ability of the test compound to interact with a CARD-3 or CARD-4 protein comprises determining the ability of the CARD-3 or CARD-4 protein to preferentially bind to or modulate the activity of a CARD-3 or CARD-4 target molecule. The cell-free assays of the present invention are amenable to use of both the soluble form or the membrane-associated form of CARD-3 or CARD-4. A membrane-associated form of CARD-3 or CARD-4 refers to CARD-3 or CARD-4 that interacts with a membrane-bound target molecule. In the case of cell-free assays comprising the membrane-associated form of CARD-3 or

- 85 -

CARD-4, it may be desirable to utilize a solubilizing agent such that the membrane-associated form of CARD-3 or CARD-4 is maintained in solution. Examples of such solubilizing agents include non-ionic detergents such as  
5 n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-114, Thesit®, Isotridecypoly(ethylene glycol ether)n, 3-[(3-cholamidopropyl)dimethylamminio]-1-propane  
10 sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate.

In more than one embodiment of the above assay  
15 methods of the present invention, it may be desirable to immobilize either CARD-3 or CARD-4 or its target molecule to facilitate separation of complexed from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of a test  
20 compound to CARD-3 or CARD-4, or interaction of CARD-3 or CARD-4 with a target molecule in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes,  
25 and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-transferase/ CARD-3 or CARD-4 fusion proteins or glutathione-S-transferase/target  
30 fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical; St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the test compound or the test compound and either the non-adsorbed target protein or CARD-3 or  
35 CARD-4 protein, and the mixture incubated under

- 86 -

conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtitre plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of CARD-3 or CARD-4 binding or activity determined using standard techniques.

10 In an alternative embodiment, MYC or HA epitope tag CARD-3 or CARD-4 fusion proteins or MYC or HA epitope tag target fusion proteins can be adsorbed onto anti-MYC or anti-HA antibody coated microbeads or onto anti-MYC or anti-HA antibody coated microtitre plates, which are then

15 combined with the test compound or the test compound and either the non-adsorbed target protein or CARD-3 or CARD-4 protein, and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following

20 incubation, the beads or microtitre plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated

25 from the matrix, and the level of CARD-3 or CARD-4 binding or activity determined using standard techniques. Example 12 describes an HA epitope tagged CARD-4 protein that physically interacts in a coimmunoprecipitation assay with MYC epitope tagged CARD-3. In an embodiment

30 of the invention, HA epitope tagged CARD-4 could be used in combination with MYC epitope CARD-3 in the sort of protein-protein interaction assay described earlier in this paragraph.

Other techniques for immobilizing proteins on

35 matrices can also be used in the screening assays of the

- 87 -

invention. For example, either CARD-3 or CARD-4 or its target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated CARD-3 or CARD-4 or target molecules can be prepared from  
5 biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals; Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with CARD-3 or CARD-4  
10 or target molecules but which do not interfere with binding of the CARD-3 or CARD-4 protein to its target molecule can be derivatized to the wells of the plate, and unbound target or CARD-3 or CARD-4 trapped in the wells by antibody conjugation. Methods for detecting  
15 such complexes, in addition to those described above for the GST-immobilized complexes and epitope tag immobilized complexes, include immunodetection of complexes using antibodies reactive with the CARD-3 or CARD-4 or target molecule, as well as enzyme-linked assays which rely on  
20 detecting an enzymatic activity associated with the CARD-3 or CARD-4 or target molecule.

In another embodiment, modulators of CARD-3 or CARD-4 expression are identified in a method in which a cell is contacted with a candidate compound and the  
25 expression of the CARD-3 or CARD-4 promoter, mRNA or protein in the cell is determined. The level of expression of CARD-3 or CARD-4 mRNA or protein in the presence of the candidate compound is compared to the level of expression of CARD-3 or CARD-4 mRNA or protein  
30 in the absence of the candidate compound. The candidate compound can then be identified as a modulator of CARD-3 or CARD-4 expression based on this comparison. For example, when expression of CARD-3 or CARD-4 mRNA or protein is greater (statistically significantly greater)  
35 in the presence of the candidate compound than in its

- 88 -

absence, the candidate compound is identified as a stimulator of CARD-3 or CARD-4 mRNA or protein expression. Alternatively, when expression of CARD-3 or CARD-4 mRNA or protein is less (statistically significantly less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of CARD-3 or CARD-4 mRNA or protein expression. The level of CARD-3 or CARD-4 mRNA or protein expression in the cells can be determined by methods described herein for detecting CARD-3 or CARD-4 mRNA or protein. The activity of the CARD-3 or CARD-4 promoter can be assayed by linking the CARD-3 or CARD-4 promoter to a reporter gene such as luciferase, secreted alkaline phosphatase, or beta-galactosidase and introducing the resulting construct into an appropriate vector, transfecting a host cell line, and measuring the activity of the reporter gene in response to test compounds. For example, two CARD-4-specific mRNAs were detected in a Northern blotting experiment, one of 4.6 kilobases and the other of 6.5-7.0 kilobases (Example 11). In Example 11, CARD-4-specific mRNA species were found to be widely distributed in the tissues and cell lines studied.

In yet another aspect of the invention, the CARD-3 or CARD-4 proteins can be used as "bait proteins" in a two-hybrid assay or three hybrid assay (see, e.g., U.S. Patent No. 5,283,317; Zervos et al. (1993) Cell 72:223-232; Madura et al. (1993) J. Biol. Chem. 268:12046-12054; Bartel et al. (1993) Bio/Techniques 14:920-924; Iwabuchi et al. (1993) Oncogene 8:1693-1696; and PCT Publication No. WO 94/10300), to identify other proteins, which bind to or interact with CARD-3 or CARD-4 ("CARD-3 or CARD-4-binding proteins" or "CARD-3 or CARD-4-bp") and modulate CARD-3 or CARD-4 activity. Such CARD-3 or CARD-4-binding proteins are also likely to be



- 89 -

involved in the propagation of signals by the CARD-3 or CARD-4 proteins as, for example, upstream or downstream elements of the CARD-3 or CARD-4 pathway. For example, Example 7 describes the construction of a two-hybrid  
5 screening bait construct including human CARD-4L amino acids 1-145 comprising the CARD domain and the use of this bait construct to screen human mammary gland and prostate gland two-hybrid libraries resulting in the identification of human CARD-3 as a CARD-4 interacting  
10 protein. In another example, Example 8 describes the construction of a two-hybrid screening bait construct including human CARD-4 amino acids 406-953 comprising the LRR domain and the use of this bait construct to screen a human mammary gland two-hybrid libraries resulting in the  
15 identification of hNUDC as a CARD-4 interacting protein.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one  
20 construct, the gene that codes for CARD-3 or CARD-4 is fused to a gene encoding the DNA binding domain of a known transcription factor (e.g., GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey"  
25 or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact, in vivo, forming an CARD-3 or CARD-4-dependent complex, the DNA-binding and activation domains of the  
30 transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (e.g., LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene  
35 can be detected and cell colonies containing the

- 90 -

functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with CARD-3 or CARD-4.

In an embodiment of the invention, the ability of  
5 a test compound to modulate the activity of CARD-3,  
CARD-4, or a biologically active portion thereof can be  
determined by assaying the ability of the test compound  
to block the binding of CARD-3 and CARD-4 to their target  
proteins in a two-hybrid system assay. Example 7  
10 describes a two-hybrid system assay for the interaction  
between CARD-3 and CARD-4 and Example 8 describes a  
two-hybrid system assay for the interaction between  
CARD-4 and its target protein hNUDC. To screen for test  
compounds that block the interaction between CARD-3 and  
15 CARD-4 and their target proteins, which include but are  
not limited to CARD-3, CARD-4, and hNUDC, a yeast  
two-hybrid screening strain coexpressing the interacting  
bait and prey constructs, for example, a CARD-4 bait  
construct and a CARD-3 prey construct as described in  
20 Example 7, is contacted with the test compound and the  
activity of the two-hybrid system reporter gene, usually  
HIS3, lacZ, or URA3 is assayed. If the strain remains  
viable but exhibits a significant decrease in reporter  
gene activity, this would indicate that the test compound  
25 has inhibited the interaction between the bait and prey  
proteins. This assay could be automated for high  
throughput drug screening purposes. In another  
embodiment of the invention, CARD-3 or CARD-4 and their  
target proteins could be configured in the reverse  
30 two-hybrid system (Vidal et al. (1996) Proc. Natl. Acad.  
Sci. USA 93:10321-6 and Vidal et al. (1996) Proc. Natl.  
Acad. Sci. USA 93:10315-20) designed specifically for  
efficient drug screening. In the reverse two-hybrid  
system, inhibition of a CARD-3 or CARD-4 physical  
35 interaction with a target protein would result in

- 91 -

induction of a reporter gene in contrast to the normal two-hybrid system where inhibition of CARD-3 or CARD-4 physical interaction with a target protein would lead to reporter gene repression. The reverse two-hybrid system  
5 is preferred for drug screening because reporter gene induction is more easily assayed than reporter gene repression.

Alternative embodiments of the invention are proteins found to physically interact with proteins that  
10 bind to CARD-3 or CARD-4. CARD-3 or CARD-4 interactors, including but not limited to hNUDC and CARD-3, could be configured into two-hybrid system baits and used in two-hybrid screens to identify additional members of the CARD-3 and CARD-4 pathway. The interactors of CARD-3 or  
15 CARD-4 interactors identified in this way could be useful targets for therapeutic intervention in CARD-4 related diseases and pathologies and an assay of their enzymatic or binding activity could be useful for the identification of test compounds that modulate CARD-3 or  
20 CARD-4 activity.

This invention further pertains to novel agents identified by the above-described screening assays and uses thereof for treatments as described herein.

#### **B. Detection Assays**

25 Portions or fragments of the cDNA sequences identified herein (and the corresponding complete gene sequences) can be used in numerous ways as polynucleotide reagents. For example, these sequences can be used to:  
(i) map their respective genes on a chromosome; and,  
30 thus, locate gene regions associated with genetic disease; (ii) identify an individual from a minute biological sample (tissue typing); and (iii) aid in forensic identification of a biological sample. These applications are described in the subsections below.

- 92 -

### 1. Chromosome Mapping

Once the sequence (or a portion of the sequence) of a gene has been isolated, this sequence can be used to map the location of the gene on a chromosome.

5 Accordingly, CARD-3 or CARD-4 nucleic acid molecules described herein or fragments thereof, can be used to map the location of CARD-3 or CARD-4 genes on a chromosome. The mapping of the CARD-3 or CARD-4 sequences to  
10 chromosomes is an important first step in correlating these sequences with genes associated with disease.

Briefly, CARD-3 or CARD-4 genes can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp in length) from the CARD-3 or CARD-4 sequences. Computer  
15 analysis of CARD-3 or CARD-4 sequences can be used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers can then be used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids  
20 containing the human gene corresponding to the CARD-3 or CARD-4 sequences will yield an amplified fragment. For example, in Example 6, human CARD-4-specific PCR primers were used to screen DNAs from a somatic cell hybrid panel showing that human CARD-4 maps to chromosome 7 close to  
25 the SHGC-31928 genetic marker.

Somatic cell hybrids are prepared by fusing somatic cells from different mammals (e.g., human and mouse cells). As hybrids of human and mouse cells grow and divide, they gradually lose human chromosomes in  
30 random order, but retain the mouse chromosomes. By using media in which mouse cells cannot grow, because they lack a particular enzyme, but human cells can, the one human chromosome that contains the gene encoding the needed enzyme, will be retained. By using various media, panels  
35 of hybrid cell lines can be established. Each cell line

- 93 -

in a panel contains either a single human chromosome or a small number of human chromosomes, and a full set of mouse chromosomes, allowing easy mapping of individual genes to specific human chromosomes. (D'Eustachio et al. 5 (1983) Science 220:919-924). Somatic cell hybrids containing only fragments of human chromosomes can also be produced by using human chromosomes with translocations and deletions.

PCR mapping of somatic cell hybrids is a rapid 10 procedure for assigning a particular sequence to a particular chromosome. Three or more sequences can be assigned per day using a single thermal cycler. Using the CARD-3 or CARD-4 sequences to design oligonucleotide primers, sublocalization can be achieved with panels of 15 fragments from specific chromosomes. Other mapping strategies which can similarly be used to map a CARD-3 or CARD-4 sequence to its chromosome include in situ hybridization (described in Fan et al. (1990) Proc. Natl. Acad. Sci. USA 87:6223-27), pre-screening with labeled 20 flow-sorted chromosomes, and pre-selection by hybridization to chromosome specific cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a DNA sequence to a metaphase chromosomal spread can further be used to provide a precise chromosomal location in one 25 step. Chromosome spreads can be made using cells whose division has been blocked in metaphase by a chemical like colcemid that disrupts the mitotic spindle. The chromosomes can be treated briefly with trypsin, and then stained with Giemsa. A pattern of light and dark bands 30 develops on each chromosome, so that the chromosomes can be identified individually. The FISH technique can be used with a DNA sequence as short as 500 or 600 bases. However, clones larger than 1,000 bases have a higher likelihood of binding to a unique chromosomal location 35 with sufficient signal intensity for simple detection.

- 94 -

Preferably 1,000 bases, and more preferably 2,000 bases will suffice to get good results at a reasonable amount of time. For a review of this technique, see Verma et al., (Human Chromosomes: A Manual of Basic Techniques  
5 (Pergamon Press, New York, 1988)).

Reagents for chromosome mapping can be used individually to mark a single chromosome or a single site on that chromosome, or panels of reagents can be used for marking multiple sites and/or multiple chromosomes.  
10 Reagents corresponding to noncoding regions of the genes actually are preferred for mapping purposes. Coding sequences are more likely to be conserved within gene families, thus increasing the chance of cross hybridizations during chromosomal mapping.

15 Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man, available on-line  
20 through Johns Hopkins University Welch Medical Library). The relationship between genes and disease, mapped to the same chromosomal region, can then be identified through linkage analysis (co-inheritance of physically adjacent genes), described in, e.g., Egeland et al. (1987) Nature,  
25 325:783-787.

Moreover, differences in the DNA sequences between individuals affected and unaffected with a disease associated with the CARD-3 or CARD-4 gene can be determined. If a mutation is observed in some or all of  
30 the affected individuals but not in any unaffected individuals, then the mutation is likely to be the causative agent of the particular disease. Comparison of affected and unaffected individuals generally involves first looking for structural alterations in the  
35 chromosomes such as deletions or translocations that are

- 95 -

visible from chromosome spreads or detectable using PCR based on that DNA sequence. Ultimately, complete sequencing of genes from several individuals can be performed to confirm the presence of a mutation and to  
5 distinguish mutations from polymorphisms.

## 2. Tissue Typing

The CARD-3 or CARD-4 sequences of the present invention can also be used to identify individuals from minute biological samples. The United States military,  
10 for example, is considering the use of restriction fragment length polymorphism (RFLP) for identification of its personnel. In this technique, an individual's genomic DNA is digested with one or more restriction enzymes, and probed on a Southern blot to yield unique  
15 bands for identification. This method does not suffer from the current limitations of "Dog Tags" which can be lost, switched, or stolen, making positive identification difficult. The sequences of the present invention are useful as additional DNA markers for RFLP (described in  
20 U.S. Patent 5,272,057).

Furthermore, the sequences of the present invention can be used to provide an alternative technique which determines the actual base-by-base DNA sequence of selected portions of an individual's genome. Thus, the  
25 CARD-3 or CARD-4 sequences described herein can be used to prepare two PCR primers from the 5' and 3' ends of the sequences. These primers can then be used to amplify an individual's DNA and subsequently sequence it.

Panels of corresponding DNA sequences from  
30 individuals, prepared in this manner, can provide unique individual identifications, as each individual will have a unique set of such DNA sequences due to allelic differences. The sequences of the present invention can be used to obtain such identification sequences from  
35 individuals and from tissue. The CARD-3 or CARD-4

- 96 -

sequences of the invention uniquely represent portions of the human genome. Allelic variation occurs to some degree in the coding regions of these sequences, and to a greater degree in the noncoding regions. It is estimated  
5 that allelic variation between individual humans occurs with a frequency of about once per each 500 bases. Each of the sequences described herein can, to some degree, be used as a standard against which DNA from an individual can be compared for identification purposes. Because  
10 greater numbers of polymorphisms occur in the noncoding regions, fewer sequences are necessary to differentiate individuals. The noncoding sequences of SEQ ID NO:1, SEQ ID NO:7, SEQ ID NO:25 and SEQ ID NO:42 can comfortably provide positive individual identification with a panel  
15 of perhaps 10 to 1,000 primers which each yield a noncoding amplified sequence of 100 bases. If predicted coding sequences, such as those in SEQ ID NO:3, SEQ ID NO:9, and SEQ ID NO:27 are used, a more appropriate number of primers for positive individual identification  
20 would be 500-2,000.

If a panel of reagents from CARD-3 or CARD-4 sequences described herein is used to generate a unique identification database for an individual, those same reagents can later be used to identify tissue from that  
25 individual. Using the unique identification database, positive identification of the individual, living or dead, can be made from extremely small tissue samples.

### 3. Use of Partial CARD-3 or CARD-4 Sequences in Forensic Biology

30 DNA-based identification techniques can also be used in forensic biology. Forensic biology is a scientific field employing genetic typing of biological evidence found at a crime scene as a means for positively identifying, for example, a perpetrator of a crime. To  
35 make such an identification, PCR technology can be used



- 97 -

to amplify DNA sequences taken from very small biological samples such as tissues, e.g., hair or skin, or body fluids, e.g., blood, saliva, or semen found at a crime scene. The amplified sequence can then be compared to a standard, thereby allowing identification of the origin of the biological sample.

The sequences of the present invention can be used to provide polynucleotide reagents, e.g., PCR primers, targeted to specific loci in the human genome, which can enhance the reliability of DNA-based forensic identifications by, for example, providing another "identification marker" (i.e. another DNA sequence that is unique to a particular individual). As mentioned above, actual base sequence information can be used for identification as an accurate alternative to patterns formed by restriction enzyme generated fragments. Sequences targeted to noncoding regions of SEQ ID NO:1, SEQ ID NO:7, and SEQ ID NO:25 are particularly appropriate for this use as greater numbers of polymorphisms occur in the noncoding regions, making it easier to differentiate individuals using this technique. Examples of polynucleotide reagents include the CARD-3 or CARD-4 sequences or portions thereof, e.g., fragments derived from the noncoding regions of SEQ ID NO:1, SEQ ID NO:7, or SEQ ID NO:25 which have a length of at least 20 or 30 bases.

The CARD-3 or CARD-4 sequences described herein can further be used to provide polynucleotide reagents, e.g., labeled or labelable probes which can be used in, for example, an in situ hybridization technique, to identify a specific tissue, e.g., brain tissue. This can be very useful in cases where a forensic pathologist is presented with a tissue of unknown origin. Panels of such CARD-3 or CARD-4 probes can be used to identify tissue by species and/or by organ type.

- 98 -

In a similar fashion, these reagents, e.g., CARD-3 or CARD-4 primers or probes can be used to screen tissue culture for contamination (i.e., screen for the presence of a mixture of different types of cells in a culture).

5           **C. Predictive Medicine**

The present invention also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, pharmacogenomics, and monitoring clinical trials are used for prognostic (predictive) purposes to thereby treat an individual prophylactically. Accordingly, one aspect of the present invention relates to diagnostic assays for determining CARD-3 or CARD-4 protein and/or nucleic acid expression as well as CARD-3 or CARD-4 activity, in the context of a biological sample (e.g., blood, serum, cells, tissue) to thereby determine whether an individual is afflicted with a disease or disorder, or is at risk of developing a disorder, associated with aberrant CARD-3 or CARD-4 expression or activity. The invention also provides for prognostic (or predictive) assays for determining whether an individual is at risk of developing a disorder associated with CARD-3 or CARD-4 protein, nucleic acid expression or activity. For example, mutations in a CARD-3 or CARD-4 gene can be assayed in a biological sample. Such assays can be used for prognostic or predictive purpose to thereby prophylactically treat an individual prior to the onset of a disorder characterized by or associated with CARD-3 or CARD-4 protein, nucleic acid expression or activity.

30           Another aspect of the invention provides methods for determining CARD-3 or CARD-4 protein, nucleic acid expression or CARD-3 or CARD-4 activity in an individual to thereby select appropriate therapeutic or prophylactic agents for that individual (referred to herein as

- 99 -

"pharmacogenomics"). Pharmacogenomics allows for the selection of agents (e.g., drugs) for therapeutic or prophylactic treatment of an individual based on the genotype of the individual (e.g., the genotype of the individual examined to determine the ability of the individual to respond to a particular agent.)

Yet another aspect of the invention pertains to monitoring the influence of agents (e.g., drugs or other compounds) on the expression or activity of CARD-3 or CARD-4 in clinical trials.

These and other agents are described in further detail in the following sections.

#### **1. Diagnostic Assays**

An exemplary method for detecting the presence or absence of CARD-3 or CARD-4 in a biological sample involves obtaining a biological sample from a test subject and contacting the biological sample with a compound or an agent capable of detecting CARD-3 or CARD-4 protein or nucleic acid (e.g., mRNA, genomic DNA) that encodes CARD-3 or CARD-4 protein such that the presence of CARD-3 or CARD-4 is detected in the biological sample. An agent for detecting CARD-3 or CARD-4 mRNA or genomic DNA is a labeled nucleic acid probe capable of hybridizing to CARD-3 or CARD-4 mRNA or genomic DNA. The nucleic acid probe can be, for example, a full-length CARD-3 or CARD-4 nucleic acid, such as the nucleic acid of SEQ ID NO: 1 or 3, SEQ ID NO: 7 or 9, SEQ ID NO:25 or 27, or a portion thereof, such as an oligonucleotide of at least 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to CARD-3 or CARD-4 mRNA or genomic DNA, or a human CARD-4 splice variant such as the nucleic acid of SEQ ID NO:38 or SEQ ID NO:40. Other suitable probes for use in the diagnostic assays of the invention are described herein. For example, Example

- 100 -

11 describes the use of a nucleic acid probe to detect CARD-4 mRNAs in human tissues and cell lines and the probe used in this experiment could be used for a diagnostic assay.

5           An agent for detecting CARD-3 or CARD-4 protein can be an antibody capable of binding to CARD-3 or CARD-4 protein, preferably an antibody with a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. For example, polypeptides corresponding to  
10 amino acids 128-139 and 287-298 of human CARD-4L were used to immunize rabbits and produce polyclonal antibodies that specifically recognize human CARD-4L. An intact antibody, or a fragment thereof (e.g., Fab or F(ab')<sub>2</sub>) can be used. The term "labeled", with regard to  
15 the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (i.e., physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is  
20 directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin. The term "biological  
25 sample" is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. That is, the detection method of the invention can be used to detect CARD-3 or CARD-4 mRNA, protein, or genomic DNA in  
30 a biological sample in vitro as well as *in vivo*. For example, *in vitro* techniques for detection of CARD-3 or CARD-4 mRNA include Northern hybridizations and *in situ* hybridizations. For example, Example 11 contains the use of a human CARD-4L nucleic acid probe for a Northern  
35 blotting analysis of mRNA species encoded by human

- 101 -

CARD-4L detected in RNA samples from human tissues and cell lines. In vitro techniques for detection of CARD-3 or CARD-4 protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence. In vitro techniques for detection of CARD-3 or CARD-4 genomic DNA include Southern hybridizations. Furthermore, in vivo techniques for detection of CARD-3 or CARD-4 protein include introducing into a subject a labeled anti-CARD-3 or CARD-4 antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

In one embodiment, the biological sample contains protein molecules from the test subject. Alternatively, the biological sample can contain mRNA molecules from the test subject or genomic DNA molecules from the test subject. An biological sample is a peripheral blood leukocyte sample isolated by conventional means from a subject.

In another embodiment, the methods further involve obtaining a control biological sample from a control subject, contacting the control sample with a compound or agent capable of detecting CARD-3 or CARD-4 protein, mRNA, or genomic DNA, such that the presence of CARD-3 or CARD-4 protein, mRNA or genomic DNA is detected in the biological sample, and comparing the presence of CARD-3 or CARD-4 protein, mRNA or genomic DNA in the control sample with the presence of CARD-3 or CARD-4 protein, mRNA or genomic DNA in the test sample.

The invention also encompasses kits for detecting the presence of CARD-3 or CARD-4 in a biological sample (a test sample). Such kits can be used to determine if a subject is suffering from or is at increased risk of developing a disorder associated with aberrant expression of CARD-3 or CARD-4 (e.g., an immunological disorder).

- 102 -

For example, the kit can comprise a labeled compound or agent capable of detecting CARD-3 or CARD-4 protein or mRNA in a biological sample and means for determining the amount of CARD-3 or CARD-4 in the sample (e.g., an  
5 anti-CARD-3 or CARD-4 antibody or an oligonucleotide probe which binds to DNA encoding CARD-3 or CARD-4, e.g., SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25 or SEQ ID NO:27). Kits may also include instruction for observing that the tested subject is  
10 suffering from or is at risk of developing a disorder associated with aberrant expression of CARD-3 or CARD-4 if the amount of CARD-3 or CARD-4 protein or mRNA is above or below a normal level.

For antibody-based kits, the kit may comprise, for  
15 example: (1) a first antibody (e.g., attached to a solid support) which binds to CARD-3 or CARD-4 protein; and, optionally, (2) a second, different antibody which binds to CARD-3 or CARD-4 protein or the first antibody and is conjugated to a detectable agent.

20 For oligonucleotide-based kits, the kit may comprise, for example: (1) a oligonucleotide, e.g., a detectably labelled oligonucleotide, which hybridizes to a CARD-3 or CARD-4 nucleic acid sequence or (2) a pair of primers useful for amplifying a CARD-3 or CARD-4 nucleic  
25 acid molecule.

The kit may also comprise, e.g., a buffering agent, a preservative, or a protein stabilizing agent. The kit may also comprise components necessary for detecting the detectable agent (e.g., an enzyme or a  
30 substrate). The kit may also contain a control sample or a series of control samples which can be assayed and compared to the test sample contained. Each component of the kit is usually enclosed within an individual container and all of the various containers are within a  
35 single package along with instructions for observing

- 103 -

whether the tested subject is suffering from or is at risk of developing a disorder associated with aberrant expression of CARD-3 or CARD-4.

## 2. Prognostic Assays

5           The methods described herein can furthermore be utilized as diagnostic or prognostic assays to identify subjects having or at risk of developing a disease or disorder associated with aberrant CARD-3 or CARD-4 expression or activity. For example, the assays  
10 described herein, such as the preceding diagnostic assays or the following assays, can be utilized to identify a subject having or at risk of developing a disorder associated with CARD-3 or CARD-4 protein, nucleic acid expression or activity. Alternatively, the prognostic  
15 assays can be utilized to identify a subject having or at risk for developing such a disease or disorder. Thus, the present invention provides a method in which a test sample is obtained from a subject and CARD-3 or CARD-4 protein or nucleic acid (e.g., mRNA, genomic DNA) is  
20 detected, wherein the presence of CARD-3 or CARD-4 protein or nucleic acid is diagnostic for a subject having or at risk of developing a disease or disorder associated with aberrant CARD-3 or CARD-4 expression or activity. As used herein, a "test sample" refers to a  
25 biological sample obtained from a subject of interest. For example, a test sample can be a biological fluid (e.g., serum), cell sample, or tissue. Furthermore, the prognostic assays described herein can be used to determine whether a subject can be administered an agent  
30 (e.g., an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate) to treat a disease or disorder associated with aberrant CARD-3 or CARD-4 expression or activity. For example, such methods can be used to determine whether a  
35 subject can be effectively treated with a specific agent

- 104 -

or class of agents (e.g., agents of a type which decrease CARD-3 or CARD-4 activity). Thus, the present invention provides methods for determining whether a subject can be effectively treated with an agent for a disorder.

5 associated with aberrant CARD-3 or CARD-4 expression or activity in which a test sample is obtained and CARD-3 or CARD-4 protein or nucleic acid is detected (e.g., wherein the presence of CARD-3 or CARD-4 protein or nucleic acid is diagnostic for a subject that can be administered the  
10 agent to treat a disorder associated with aberrant CARD-3 or CARD-4 expression or activity).

The methods of the invention can also be used to detect genetic lesions or mutations in a CARD-3 or CARD-4 gene, thereby determining if a subject with the lesioned  
15 gene is at risk for a disorder characterized by aberrant cell proliferation and/or differentiation. In preferred embodiments, the methods include detecting, in a sample of cells from the subject, the presence or absence of a genetic lesion characterized by at least one of an  
20 alteration affecting the integrity of a gene encoding a CARD-3 or CARD-4-protein, or the mis-expression of the CARD-3 or CARD-4 gene. For example, such genetic lesions can be detected by ascertaining the existence of at least one of 1) a deletion of one or more nucleotides from a  
25 CARD-3 or CARD-4 gene; 2) an addition of one or more nucleotides to a CARD-3 or CARD-4 gene; 3) a substitution of one or more nucleotides of a CARD-3 or CARD-4 gene, 4) a chromosomal rearrangement of a CARD-3 or CARD-4 gene; 5) an alteration in the level of a messenger RNA  
30 transcript of a CARD-3 or CARD-4 gene, 6) aberrant modification of a CARD-3 or CARD-4 gene, such as of the methylation pattern of the genomic DNA, 7) the presence of a non-wild type splicing pattern of a messenger RNA transcript of a CARD-3 or CARD-4 gene (e.g, caused by a  
35 mutation in a splice donor or splice acceptor site), 8) a



- 105 -

non-wild type level of a CARD-3 or CARD-4-protein, 9)  
allelic loss of a CARD-3 or CARD-4 gene, and 10)  
inappropriate post-translational modification of a CARD-3  
or CARD-4-protein. As described herein, there are a  
5 large number of assay techniques known in the art which  
can be used for detecting lesions in a CARD-3 or CARD-4  
gene. A biological sample is a peripheral blood  
leukocyte sample isolated by conventional means from a  
subject.

10 In certain embodiments, detection of the lesion  
involves the use of a probe/primer in a polymerase chain  
reaction (PCR) (see, e.g., U.S. Patent Nos. 4,683,195 and  
4,683,202), such as anchor PCR or RACE PCR, or,  
alternatively, in a ligation chain reaction (LCR) (see,  
15 e.g., Landegran et al. (1988) Science 241:1077-1080; and  
Nakazawa et al. (1994) Proc. Natl. Acad. Sci. USA  
91:360-364), the latter of which can be particularly  
useful for detecting point mutations in the CARD-3 or  
CARD-4-gene (see, e.g., Abravaya et al. (1995) Nucleic  
20 Acids Res. 23:675-682). This method can include the  
steps of collecting a sample of cells from a patient,  
isolating nucleic acid (e.g., genomic, mRNA or both) from  
the cells of the sample, contacting the nucleic acid  
sample with one or more primers which specifically  
25 hybridize to a CARD-3 or CARD-4 gene under conditions  
such that hybridization and amplification of the CARD-3  
or CARD-4-gene (if present) occurs, and detecting the  
presence or absence of an amplification product, or  
detecting the size of the amplification product and  
30 comparing the length to a control sample. It is  
anticipated that PCR and/or LCR may be desirable to use  
as a preliminary amplification step in conjunction with  
any of the techniques used for detecting mutations  
described herein.

- 106 -

Alternative amplification methods include: self sustained sequence replication (Guatelli et al. (1990) Proc. Natl. Acad. Sci. USA 87:1874-1878), transcriptional amplification system (Kwoh, et al. (1989) Proc. Natl. Acad. Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi et al. (1988) Bio/Technology 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In an alternative embodiment, mutations in a CARD-3 or CARD-4 gene from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence specific ribozymes (see, e.g., U.S. Patent No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

In other embodiments, genetic mutations in CARD-3 or CARD-4 can be identified by hybridizing a sample and control nucleic acids, e.g., DNA or RNA, to high density arrays containing hundreds or thousands of oligonucleotide probes (Cronin et al. (1996) Human Mutation 7:244-255; Kozal et al. (1996) Nature Medicine 2:753-759). For example, genetic mutations in CARD-3 or CARD-4 can be identified in two-dimensional arrays containing light-generated DNA probes as described in Cronin et al. supra. Briefly, a first hybridization

- 107 -

array of probes can be used to scan through long stretches of DNA in a sample and control to identify base changes between the sequences by making linear arrays of sequential overlapping probes. This step allows the  
5 identification of point mutations. This step is followed by a second hybridization array that allows the characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of  
10 parallel probe sets, one complementary to the wild-type gene and the other complementary to the mutant gene.

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the CARD-3 or CARD-4 gene and detect  
15 mutations by comparing the sequence of the sample CARD-3 or CARD-4 with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on techniques developed by Maxam and Gilbert ((1977) Proc. Natl. Acad. Sci. USA 74:560) or Sanger  
20 ((1977) Proc. Natl. Acad. Sci. USA 74:5463). It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays ((1995) Bio/Techniques 19:448), including sequencing by mass spectrometry (see, e.g., PCT  
25 Publication No. WO 94/16101; Cohen et al. (1996) Adv. Chromatogr. 36:127-162; and Griffin et al. (1993) Appl. Biochem. Biotechnol. 38:147-159).

Other methods for detecting mutations in the CARD-3 or CARD-4 gene include methods in which protection  
30 from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers et al. (1985) Science 230:1242). In general, the art technique of "mismatch cleavage" starts by providing heteroduplexes of formed by hybridizing (labeled) RNA or DNA containing the  
35 wild-type CARD-3 or CARD-4 sequence with potentially

- 108 -

mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to basepair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with S1 nuclease to enzymatically digesting the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels to determine the site of mutation. See, e.g., Cotton et al (1988) Proc. Natl Acad Sci USA 85:4397; Saleeba et al (1992) Methods Enzymol. 217:286-295. In an embodiment, the control DNA or RNA can be labeled for detection.

In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes) in defined systems for detecting and mapping point mutations in CARD-3 or CARD-4 cDNAs obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu et al. (1994) Carcinogenesis 15:1657-1662). According to an exemplary embodiment, a probe based on a CARD-3 or CARD-4 sequence, e.g., a wild-type CARD-3 or CARD-4 sequence, is hybridized to a cDNA or other DNA product from a test cell(s). The duplex is treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like. See, e.g., U.S. Patent No. 5,459,039.

- 109 -

In other embodiments, alterations in electrophoretic mobility will be used to identify mutations in CARD-3 or CARD-4 genes. For example, single strand conformation polymorphism (SSCP) may be used to  
5 detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita et al. (1989) Proc Natl. Acad. Sci USA: 86:2766, see also Cotton (1993) Mutat. Res. 285:125-144; and Hayashi (1992) Genet Anal Tech Appl 9:73-79). Single-stranded DNA fragments of  
10 sample and control CARD-3 or CARD-4 nucleic acids will be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a  
15 single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In an embodiment, the subject method  
20 utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen et al. (1991) Trends Genet 7:5).

In yet another embodiment, the movement of mutant  
25 or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers et al. (1985) Nature 313:495). When DGGE is used as the method of analysis, DNA will be modified to insure that it does not  
30 completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and  
35 Reissner (1987) Biophys Chem 265:12753).

- 110 -

Examples of other techniques for detecting point mutations include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example,

5 oligonucleotide primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki et al. (1986) Nature 324:163); Saiki et al. (1989) Proc. Natl Acad. Sci  
10 USA 86:6230). Such allele specific oligonucleotides are hybridized to PCR amplified target DNA or a number of different mutations when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA.

15 Alternatively, allele specific amplification technology which depends on selective PCR amplification may be used in conjunction with the instant invention. Oligonucleotides used as primers for specific amplification may carry the mutation of interest in the  
20 center of the molecule (so that amplification depends on differential hybridization) (Gibbs et al. (1989) Nucleic Acids Res. 17:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner (1993)  
25 Tibtech 11:238). In addition, it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection (Gasparini et al. (1992) Mol. Cell Probes 6:1). It is anticipated that in certain embodiments amplification may also be  
30 performed using Taq ligase for amplification (Barany (1991) Proc. Natl. Acad. Sci USA 88:189). In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect the presence of a known mutation at a specific

- 111 -

site by looking for the presence or absence of amplification.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe nucleic acid or antibody reagent described herein, which may be conveniently used, e.g., in clinical settings to diagnose patients exhibiting symptoms or family history of a disease or illness involving a CARD-3 or CARD-4 gene.

Furthermore, any cell type or tissue, preferably peripheral blood leukocytes, in which CARD-3 or CARD-4 is expressed may be utilized in the prognostic assays described herein.

### 3. Pharmacogenomics

Agents, or modulators which have a stimulatory or inhibitory effect on CARD-3 or CARD-4 activity (e.g., CARD-3 or CARD-4 gene expression) as identified by a screening assay described herein can be administered to individuals to treat (prophylactically or therapeutically) disorders (e.g., an immunological disorder) associated with aberrant CARD-3 or CARD-4 activity. In conjunction with such treatment, the pharmacogenomics (i.e., the study of the relationship between an individual's genotype and that individual's response to a foreign compound or drug) of the individual may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, the pharmacogenomics of the individual permits the selection of effective agents (e.g., drugs) for prophylactic or therapeutic treatments based on a consideration of the individual's genotype. Such pharmacogenomics can further be used to determine appropriate dosages and therapeutic regimens.

- 112 -

Accordingly, the activity of CARD-3 or CARD-4 protein, expression of CARD-3 or CARD-4 nucleic acid, or mutation content of CARD-3 or CARD-4 genes in an individual can be determined to thereby select appropriate agent(s) for  
5 therapeutic or prophylactic treatment of the individual.

Pharmacogenomics deals with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See, e.g., Linder (1997) Clin. Chem.  
10 43(2):254-266. In general, two types of pharmacogenetic conditions can be differentiated. Genetic conditions transmitted as a single factor altering the way drugs act on the body (altered drug action) or genetic conditions transmitted as single factors altering the way the body  
15 acts on drugs (altered drug metabolism). These pharmacogenetic conditions can occur either as rare defects or as polymorphisms. For example, glucose-6-phosphate dehydrogenase deficiency (G6PD) is a common inherited enzymopathy in which the main clinical  
20 complication is haemolysis after ingestion of oxidant drugs (anti-malarials, sulfonamides, analgesics, nitrofurans) and consumption of fava beans.

As an illustrative embodiment, the activity of drug metabolizing enzymes is a major determinant of both  
25 the intensity and duration of drug action. The discovery of genetic polymorphisms of drug metabolizing enzymes (e.g., N-acetyltransferase 2 (NAT 2) and cytochrome P450 enzymes CYP2D6 and CYP2C19) has provided an explanation as to why some patients do not obtain the expected drug  
30 effects or show exaggerated drug response and serious toxicity after taking the standard and safe dose of a drug. These polymorphisms are expressed in two phenotypes in the population, the extensive metabolizer (EM) and poor metabolizer (PM). The prevalence of PM is  
35 different among different populations. For example, the



- 113 -

gene coding for CYP2D6 is highly polymorphic and several mutations have been identified in PM, which all lead to the absence of functional CYP2D6. Poor metabolizers of CYP2D6 and CYP2C19 quite frequently experience  
5 exaggerated drug response and side effects when they receive standard doses. If a metabolite is the active therapeutic moiety, PM show no therapeutic response, as demonstrated for the analgesic effect of codeine mediated by its CYP2D6-formed metabolite morphine. The other  
10 extreme are the so called ultra-rapid metabolizers who do not respond to standard doses. Recently, the molecular basis of ultra-rapid metabolism has been identified to be due to CYP2D6 gene amplification.

Thus, the activity of CARD-3 or CARD-4 protein,  
15 expression of CARD-3 or CARD-4 nucleic acid, or mutation content of CARD-3 or CARD-4 genes in an individual can be determined to thereby select appropriate agent(s) for therapeutic or prophylactic treatment of the individual. In addition, pharmacogenetic studies can be used to apply  
20 genotyping of polymorphic alleles encoding drug-metabolizing enzymes to the identification of an individual's drug responsiveness phenotype. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus  
25 enhance therapeutic or prophylactic efficiency when treating a subject with a CARD-3 or CARD-4 modulator, such as a modulator identified by one of the exemplary screening assays described herein.

#### 4. Monitoring of Effects During Clinical Trials

30 Monitoring the influence of agents (e.g., drugs, compounds) on the expression or activity of CARD-3 or CARD-4 (e.g., the ability to modulate aberrant cell proliferation and/or differentiation) can be applied not only in basic drug screening, but also in clinical  
35 trials. For example, the effectiveness of an agent

- 114 -

determined by a screening assay as described herein to increase CARD-3 or CARD-4 gene expression, protein levels, or upregulate CARD-3 or CARD-4 activity, can be monitored in clinical trials of subjects exhibiting  
5 decreased CARD-3 or CARD-4 gene expression, protein levels, or downregulated CARD-3 or CARD-4 activity. Alternatively, the effectiveness of an agent determined by a screening assay to decrease CARD-3 or CARD-4 gene  
10 expression, protein levels, or downregulated CARD-3 or CARD-4 activity, can be monitored in clinical trials of subjects exhibiting increased CARD-3 or CARD-4 gene expression, protein levels, or upregulated CARD-3 or  
CARD-4 activity. In such clinical trials, the expression or activity of CARD-3 or CARD-4 and, preferably, other  
15 genes that have been implicated in, for example, a cellular proliferation disorder can be used as a "read out" or markers of the immune responsiveness of a particular cell.

For example, and not by way of limitation, genes,  
20 including CARD-3 or CARD-4, that are modulated in cells by treatment with an agent (e.g., compound, drug or small molecule) which modulates CARD-3 or CARD-4 activity (e.g., identified in a screening assay as described  
herein) can be identified. Thus, to study the effect of  
25 agents on cellular proliferation disorders, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of CARD-3 or CARD-4 and other genes implicated in the  
disorder. The levels of gene expression (i.e., a gene  
30 expression pattern) can be quantified by Northern blot analysis or RT-PCR, as described herein, or alternatively by measuring the amount of protein produced, by one of the methods as described herein, or by measuring the  
levels of activity of CARD-3 or CARD-4 or other genes.  
35 In this way, the gene expression pattern can serve as a

- 115 -

marker, indicative of the physiological response of the cells to the agent. Accordingly, this response state may be determined before, and at various points during, treatment of the individual with the agent.

5           In an embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (e.g., an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate identified by the  
10 screening assays described herein) comprising the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level of expression of a CARD-3 or CARD-4 protein, mRNA, or genomic DNA in the preadministration  
15 sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the CARD-3 or CARD-4 protein, mRNA, or genomic DNA in the post-administration samples; (v) comparing the level of expression or activity of the  
20 CARD-3 or CARD-4 protein, mRNA, or genomic DNA in the pre-administration sample with the CARD-3 or CARD-4 protein, mRNA, or genomic DNA in the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example,  
25 increased administration of the agent may be desirable to increase the expression or activity of CARD-3 or CARD-4 to higher levels than detected, i.e., to increase the effectiveness of the agent. Alternatively, decreased administration of the agent may be desirable to decrease  
30 expression or activity of CARD-3 or CARD-4 to lower levels than detected, i.e., to decrease the effectiveness of the agent.

- 116 -

### C. Methods of Treatment

The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or  
5 having a disorder associated with aberrant CARD-3 or CARD-4 expression or activity.

#### 1. Prophylactic Methods

In one aspect, the invention provides a method for preventing in a subject, a disease or condition  
10 associated with an aberrant CARD-3 or CARD-4 expression or activity, by administering to the subject an agent which modulates CARD-3 or CARD-4 expression or at least one CARD-3 or CARD-4 activity. Subjects at risk for a disease which is caused or contributed to by aberrant  
15 CARD-3 or CARD-4 expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the CARD-3 or CARD-4  
20 aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of CARD-3 or CARD-4 aberrancy, for example, a CARD-3 or CARD-4 agonist or CARD-3 or CARD-4 antagonist agent can be used for treating the subject. The  
25 appropriate agent can be determined based on screening assays described herein. Activities of CARD-3 or CARD-4 that could be modulated for prophylactic purposes include, but are not limited to, 1) CARD-3 or CARD-4 gene or protein expression, for example, see Example 11 for a  
30 description of the mRNA expression pattern of human CARD-4; 2) CARD-3 or CARD-4 binding to a target protein, for example, see Examples 7, 8, and 12 for a description of proteins known to bind to CARD-3 or CARD-4; 3) CARD-4 regulation of NF- $\kappa$ B as described in Example 9; and 4)

- 117 -

CARD-3 and CARD-4 enhancement of caspase 9 activity as described in Example 10.

## 2. Therapeutic Methods

Another aspect of the invention pertains to  
5 methods of modulating CARD-3 or CARD-4 expression or activity for therapeutic purposes. The modulatory method of the invention involves contacting a cell with an agent that modulates one or more of the activities of CARD-3 or CARD-4 protein activity associated with the cell. An  
10 agent that modulates CARD-3 or CARD-4 protein activity can be an agent as described herein, such as a nucleic acid or a protein, a naturally-occurring cognate ligand of a CARD-3 or CARD-4 protein, a peptide, a CARD-3 or CARD-4 peptidomimetic, or other small molecule. In one  
15 embodiment, the agent stimulates one or more of the biological activities of CARD-3 or CARD-4 protein. Examples of such stimulatory agents include active CARD-3 or CARD-4 protein and a nucleic acid molecule encoding CARD-3 or CARD-4 that has been introduced into the cell.  
20 In another embodiment, the agent inhibits one or more of the biological activities of CARD-3 or CARD-4 protein. Examples of such inhibitory agents include antisense CARD-3 or CARD-4 nucleic acid molecules and anti-CARD-3 or CARD-4 antibodies. These modulatory methods can be  
25 performed in vitro (e.g., by culturing the cell with the agent) or, alternatively, in vivo (e.g., by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder characterized by aberrant  
30 expression or activity of a CARD-3 or CARD-4 protein or nucleic acid molecule. In one embodiment, the method involves administering an agent (e.g., an agent identified by a screening assay described herein), or combination of agents that modulates (e.g., upregulates  
35 or downregulates) CARD-3 or CARD-4 expression or

- 118 -

activity. In another embodiment, the method involves administering a CARD-3 or CARD-4 protein or nucleic acid molecule as therapy to compensate for reduced or aberrant CARD-3 or CARD-4 expression or activity. Activities of  
5 CARD-3 or CARD-4 that could be modulated for therapeutic purposes include, but are not limited to, 1) CARD-3 or CARD-4 gene or protein expression, for example, see Example 11 for a description of the mRNA expression pattern of human CARD-4; 2) CARD-3 or CARD-4 binding to a  
10 target protein, for example, see Examples 7, 8, and 12 for a description of proteins known to bind to CARD-3 or CARD-4; 3) CARD-4 regulation of NF- $\kappa$ B as described in Example 9; and 4) CARD-4 enhancement of caspase 9 activity as described in Example 10.

15 Stimulation of CARD-3 or CARD-4 activity is desirable in situations in which CARD-3 or CARD-4 is abnormally downregulated and/or in which increased CARD-3 or CARD-4 activity is likely to have a beneficial effect. Conversely, inhibition of CARD-3 or CARD-4 activity is  
20 desirable in situations in which CARD-3 or CARD-4 is abnormally upregulated, e.g., in myocardial infarction, and/or in which decreased CARD-3 or CARD-4 activity is likely to have a beneficial effect. Since CARD-4 may play be involved in the processing of cytokines,  
25 inhibiting the activity or expression CARD4- may be beneficial in patients that have aberrant inflammation.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and  
30 published patent applications cited throughout this application are hereby incorporated by reference.

- 119 -

### EXAMPLES

#### **Example 1: Isolation and Characterization of full-length Human CARD-3 and CARD-4L/S cDNAs.**

A profile of known CARD domains was used to search  
5 databases of cDNA sequences and partial cDNA sequences  
using TBLASTN (Washington University; version 2.0,  
BLOSUM62 search matrix). This search led to the  
identification of CARD-3. Using CARD-3 to search  
databases of cDNA sequences and partial cDNA sequences,  
10 another potential CARD cDNA was found. This cDNA  
sequence was used screen a human umbilical vein  
endothelial library (HUVe) and a clone containing the  
partial CARD-4S was identified. The human umbilical vein  
endothelial library was then rescreened using a probe  
15 designed against the partial CARD-4S sequence and a clone  
containing the CARD-4L sequence was identified.

#### **Example 2: Characterization of CARD-3 AND CARD-4L/S Proteins.**

In this example, the predicted amino acid  
20 sequences of human CARD-3 and CARD-4L/S proteins were  
compared to amino acid sequences of known proteins and  
various motifs were identified. For example, the CARD  
domains of CARD-3 and CARD-4 were aligned (Figure 7) with  
the CARD domains of ARC-CARD (SEQ ID NO:31), cIAP1-CARD  
25 (SEQ ID NO:32) and cIAP2-CARD (SEQ ID NO:33). In  
addition, the molecular weight of the human CARD-3 and  
CARD-4L/S proteins were predicted.

The human CARD-3 cDNA was isolated as described  
above (Figure 1; SEQ ID NO:1) and encodes a 540 amino  
30 acid protein (Figure 2: SEQ ID NO:2). CARD-3 also  
includes one predicted kinase domain (amino acid 1 to  
amino acid 300 of SEQ ID NO:2; SEQ ID NO:4), which is  
followed by a predicted linker domain (amino acid 301 to  
amino acid 431 of SEQ ID NO:2; SEQ ID NO:5) and a

- 120 -

predicted CARD domain (amino acid 432 to amino acid 540 of SEQ ID NO:2; SEQ ID NO:6).

The human CARD-4L cDNA was isolated as described above (Figure 3; SEQ ID NO:7) and has a 2859 nucleotide  
5 open reading frame (nucleotides 245-3103 of SEQ ID NO:7; SEQ ID NO:9) which encodes a 953 amino acid protein (Figure 4; SEQ ID NO:8). CARD-4L protein has a predicted CARD domain (amino acids 15-114; SEQ ID NO:10). CARD-4L is also predicted to have a nucleotide binding domain  
10 which extends from about amino acid 198 to about amino acid 397 of SEQ ID NO:8; SEQ ID NO:11, a predicted Walker Box "A", which extends from about amino acid 202 to about amino acid 209 of SEQ ID NO:8; SEQ ID NO:12, a predicted Walker Box "B", which extends from about amino acid 280  
15 to about amino acid 284, of SEQ ID NO:8; SEQ ID NO:13, a predicted kinase 1a (P-loop) domain, which extends from about amino acid 197 to about amino acid 212 of SEQ ID NO:8; SEQ ID NO:46, a predicted kinase 2 domain, which extends from about amino acid 273 to about amino acid 288  
20 of SEQ ID NO:8; SEQ ID NO:47, a predicted kinase 3a subdomain, which extends from about amino acid 327 to about amino acid 338 of SEQ ID NO:8; SEQ ID NO:14, ten predicted Leucine-rich repeats which extend from about amino acid 674 to about amino acid 950 of SEQ ID NO:8.  
25 The first Leucine-rich repeat is predicted to extend from about amino acid 674 to about amino acid 701 of SEQ ID NO:8; SEQ ID NO:15. The second Leucine-rich repeat is predicted to extend from about amino acid 702 to about amino acid 727 of SEQ ID NO:8; SEQ ID NO:16. The third  
30 Leucine-rich repeat is predicted to extend from about amino acid 728 to about amino acid 754 of SEQ ID NO:8; SEQ ID NO:17. The fourth Leucine-rich repeat is predicted to extend from about amino acid 755 to about amino acid 782 of SEQ ID NO:8; SEQ ID NO:18. The fifth  
35 Leucine-rich repeat is predicted to extend from about



- 121 -

amino acid 783 to about amino acid 810 of SEQ ID NO:8; SEQ ID NO:19. The sixth Leucine-rich repeat is predicted to extend from about amino acid 811 to about amino acid 838 of SEQ ID NO:8; SEQ ID NO:20. The seventh

5 Leucine-rich repeat is predicted to extend from about amino acid 839 to about amino acid 866 of SEQ ID NO:8; SEQ ID NO:21. The eighth Leucine-rich repeat is predicted to extend from about amino acid 867 to about amino acid 894 of SEQ ID NO:8; SEQ ID NO:22. The ninth

10 Leucine-rich repeat is predicted to extend from about amino acid 895 to about amino acid 922 of SEQ ID NO:8; SEQ ID NO:23 and the tenth leucine-rich repeat is predicted to extend from about amino acid 923 to about amino acid 950 of SEQ ID NO:8; SEQ ID NO:24.

15 The human partial CARD-4S cDNA isolated as described above (Figure 5; SEQ ID NO:25) encodes a 490 amino acid protein (Figure 6; SEQ ID NO:26). CARD-4S includes one predicted partial CARD domain (amino acids 1-74 of SEQ ID NO:26). CARD-4S is also predicted to have

20 a P-Loop which extends from about amino acid 163 to about amino acid 170 of SEQ ID NO:26; SEQ ID NO:29, and a predicted Walker Box "B" which extends from about amino acid 241 to about amino acid 245 of SEQ ID NO:26; SEQ ID NO:30.

25 A plot showing the predicted structural features of CARD-4L is presented in Figure 8. This figure shows the predicted alpha regions (Garnier-Robinson and Chou-Fasman), the predicted beta regions (Garnier-Robinson and Chou-Fasman), the predicted turn

30 regions (Garnier-Robinson and Chou-Fasman) and the predicted coil regions (Garnier-Robinson and Chou-Fasman). Also included in the figure is a hydrophilicity plot (Kyte-Doolittle), the predicted alpha and beta-amphipathic regions (Eisenberg), the predicted

35 flexible regions (Karplus-Schulz), the predicted

- 122 -

antigenic index (Jameson-Wolf) and the predicted surface probability plot (Emini).

A plot showing the predicted structural features of CARD-4S is also presented in Figure 9. This figure shows the predicted alpha regions (Garnier-Robinson and Chou-Fasman), the predicted beta regions (Garnier-Robinson and Chou-Fasman), the predicted turn regions (Garnier-Robinson and Chou-Fasman) and the predicted coil regions (Garnier-Robinson and Chou-Fasman). Also included in the figure is a hydrophilicity plot (Kyte-Doolittle), the predicted alpha and beta-amphatic regions (Eisenberg), the predicted flexible regions (Karplus-Schulz), the predicted antigenic index (Jameson-Wolf) and the predicted surface probability plot (Emini).

The predicted MW of CARD-3 is approximately 61 kDa. The predicted MW of CARD-4L is approximately 108 kDa.

### **Example 3: Preparation of CARD-3 and CARD-4 Proteins**

Recombinant CARD-3 and CARD-4 can be produced in a variety of expression systems. For example, the CARD-3 and CARD-4 peptides can be expressed as a recombinant glutathione-S-transferase (GST) fusion protein in *E. coli* and the fusion protein can be isolated and characterized. Specifically, as described above, CARD-3 or CARD-4 can be fused to GST and the fusion protein can be expressed in *E. coli* strain PEB199. Expression of the GST-CARD-3 or GST-CARD-4 fusion protein in PEB199 can be induced with IPTG. The recombinant fusion protein can be purified from crude bacterial lysates of the induced PEB199 strain by affinity chromatography on glutathione beads.

- 123 -

**Example 4: Identification of splice variants of CARD4.**

The 5' untranslated sequence from CARD-4L was used to search databases of cDNA sequences and partial cDNA sequences using BLASTN (Washington University; version 5 2.0, BLOSUM62 search matrix) for additional CARD-4 cDNA clones. This search led to the identification of two cDNA clones, clone Z from a human lymph node library and the Y clone from a human brain cDNA library. Both clones were sequenced and found to represent probable splice 10 variants of CARD-4 that encode truncated CARD-4 proteins, Y encoding a 249 amino acid protein and Z encoding a 164 amino acid protein. Fig. 10 shows the nucleotide (SEQ ID NO:38) and Fig. 11 the predicted amino acid (SEQ ID NO:39) sequences of human CARD-4Y; Fig. 12 shows the 15 nucleotide (SEQ ID NO:40) and Fig. 13 the amino acid (SEQ ID NO:41) sequences of human CARD-4Z; and Fig. 14 shows an alignment of the CARD-4L, CARD-4Y, and CARD-4Z amino acid sequences generated by the Clustal program using a PAM250 residue weight table.

20 **Example 5: Identification of murine CARD-4.**

The CARD-4 polypeptide sequence was used to search databases of cDNA sequences and partial cDNA sequences using the TBLASTN program (version 1.4, BLOSUM62 search 25 matrix, and a word length of 3) for murine CARD-4 cDNA clones. This search led to the identification of a partial murine CARD-4 clone designated murine CARD-4L. The rapid identification of cDNA ends procedure (RACE) was applied to the 5' end of the murine CARD-4L clone to elucidate the 5' end of the murine CARD-4L cDNA. Fig. 15 30 shows the murine CARD-4L nucleotide sequence (SEQ ID NO:42), Figure 16 shows the murine CARD-4L amino acid sequence (SEQ ID NO:43), and Fig. 17 shows an alignment of the murine CARD-4L and human CARD-4L amino acid

- 124 -

sequences generated by the Clustal program using a PAM250 residue weight table.

**Example 6: Identification of the chromosomal location of human CARD-4.**

5 To determine the chromosomal location of the human CARD-4 gene, the polymerase chain reaction carried out with human CARD-4-specific primers card4t, with the 5' to 3' sequence agaaggtctggtcggcaaa (SEQ ID NO:44), and card4k, with the 5' to 3' sequence aagccctgagtggaagca  
10 (SEQ ID NO:45), was used to screen DNAs from a commercially available somatic cell hybrid panel. This analysis showed that human CARD-4 maps to chromosome 7 close to the SHGC-31928 genetic marker.

**Example 7: Identification of CARD-3 in a yeast**  
15 **two-hybrid screen for proteins that physically interact with the CARD domain of human CARD-4.**

DNA encoding amino acids 1-145 of human CARD-4 comprising the CARD domain was cloned into a yeast two-hybrid screening vector to create a CARD-4,1-145-GAL4  
20 DNA-binding domain fusion for two-hybrid screening. The CARD-4,1-145-GAL4 DNA-binding domain fusion was used to screen human mammary gland and human prostate two-hybrid libraries for gene products that could physically associate with CARD-4,1-145. Twelve library plasmids  
25 expressing CARD4,1-145 interacting proteins were found to contain the CARD-domain containing protein CARD-3 thus establishing a direct or indirect physical interaction between CARD-4 and CARD-3.

In addition, DNA encoding amino acids 435-540 of  
30 CARD-3 comprising the CARD domain of CARD-3 (SEQ ID NO:6) was cloned into a yeast two-hybrid GAL4 transcriptional activation domain fusion vector to create a CARD-3,435-540-GAL4 transcriptional activation domain fusion. To

- 125 -

test whether the CARD domain of CARD-3 binds CARD-4,1-145, the CARD-3,435-540-GAL4 transcriptional activation domain fusion expression vector and the CARD-4,1-145-GAL4 DNA-binding domain fusion vector were  
5 cotransformed into a two-hybrid screening *Saccharomyces cerevisiae* (yeast) strain. The resulting cotransformed yeast strain expressed the two reporter genes that indicate a physical interaction between the two hybrid proteins in the experiment, in this case, the CARD-3,435-  
10 540-GAL4 transcriptional activation domain fusion protein and the CARD-4,1-145-GAL4 DNA-binding domain fusion protein. This experiment established a physical interaction between the CARD domain of CARD-3 and the CARD domain of CARD-4.

15 **Example 8: Identification of hNUDC in a yeast two-hybrid screen for proteins that physically interact with the LRR domain of human CARD-4.**

DNA encoding amino acids 406-953 of human CARD-4L comprising the LRR domain was cloned into a yeast  
20 two-hybrid screening vector to create a CARD-4,406-953-GAL4 DNA-binding domain fusion for two-hybrid screening. The CARD-4,406-953-GAL4 DNA-binding domain fusion was used to screen a human mammary gland two-hybrid library for gene products that  
25 could physically associate with CARD-4,406-953. One library plasmid expressing a CARD4,406-953 interacting protein was found to contain the hNUDC protein, the human ortholog of the rat NUDC protein that has been implicated in nuclear movement (Morris et al., Curr. Biol. 8:603  
30 [1998], Morris et al., Exp. Cell Res. 238:23 [1998]), thus establishing a physical interaction between CARD-4 and hNUDC.

- 126 -

**Example 9: Discovery of regulation by CARD-4 of NF- $\kappa$ B.**

The first group of experiments described in this Example were carried out to determine if CARD-4 can activate the NF- $\kappa$ B pathway. CARD-4 regulation of the  
5 NF- $\kappa$ B pathway is of interest because the NF- $\kappa$ B pathway is involved in many diseases described in (New England Journal of Medicine 336:1066 [1997]) and (American Journal of Cardiology 76:18C [1995]) and other references known to those skilled in the art. Participation of  
10 CARD-4 in the NF- $\kappa$ B pathway would make CARD-4 an attractive target for drugs that modulate the NF- $\kappa$ B pathway for treatment of NF- $\kappa$ B pathway-dependent diseases, conditions, and biological processes.

The first group of experiments showed specific  
15 CARD-4-mediated NF- $\kappa$ B pathway induction.

The second group of experiments described in this Example were carried out to determine if CARD-3, the NIK serine/threonine protein kinase (Su et al., EMBO J. 16:1279 [1997]), or the signal transduction protein TRAF6  
20 (Cao et al., Nature 383:443 [1996]), proteins known to participate in the induction of NF- $\kappa$ B (McCarthy et al., J. Biol. Chem. 273:16968 [1998]), are involved in transducing the CARD-4-dependent NF- $\kappa$ B pathway induction signal. It was found that CARD-3, NIK, and TRAF6 are all  
25 involved in transducing the CARD-4-mediated NF- $\kappa$ B pathway induction signal.

In nine transfection experiments, 293T cells coexpressing an NF- $\kappa$ B reporter plasmid and either pCI, pCI-CARD-4L (expressing CARD-4L), pCI-CARD-4S (expressing  
30 CARD-4S), pCI-APAF1 (expressing Apaf-1), pCI-APAFS (expressing an Apaf-1 variant lacking WD repeats), pCI-CARD-4LnoCARD (expressing CARD-4L without a CARD domain), pCI-CARD4LnoLRR (expressing CARD-4L without a LRR), pCI-CARD4LCARDonly (expressing CARD-4L CARD domain  
35 only), or pCI-CARD4NBSoonly (expressing CARD-4L nucleotide

- 127 -

binding sequence only) were created. 293T cells were plated in 6-well plates (35 mm wells) and transfected 2 days later (90% confluency) with 1  $\mu$ g of NF- $\kappa$ B luciferase reporter plasmid (pNF- $\kappa$ B-Luc, 5 Stratagene), 200 ng of pCMV  $\beta$ -gal, 600 ng of pCI vector and 200 ng of indicated expression plasmids using SuperFect transfection reagent (Qiagen). For dominant-negative experiments, 2 ng of CARD4 expressing plasmid and 800 ng of dominant-negative plasmid were used. Cells 10 were harvested 48 h after transfection and luciferase activity in 1000-fold diluted cell extracts was determined using the Luciferase Assay System (Promega). In addition,  $\beta$ -galactosidase activities were determined and used to normalize transfection efficiency.

15           Relative luciferase activity was determined at the end of the experiment to assess NF- $\kappa$ B pathway activation by the gene expressed by the pCI-based plasmid in each transfected cell line. The cell lines containing pCI, pCI-APAFS, pCI-APAF $\Delta$ L, pCI-CARD-4LnoCARD, and pCI- 20 CARD4NB $\Delta$ Only had similar baseline levels of luciferase expression but the cell lines containing pCI-CARD-4L, pCI-CARD4LnoLRR, and pCI-CARD4LCARD $\Delta$ Only had luciferase expression about nine fold elevated relative to baseline and the cell line containing pCI-CARD4S had luciferase 25 expression sixteen fold elevated relative to baseline. This result demonstrates induction by CARD-4S and CARD-4L of the NF- $\kappa$ B pathway. This CARD-4 mediated NF- $\kappa$ B pathway induction is dependent on the CARD-4 CARD domain because the pCI-CARD-4noCARD construct expressing CARD-4 lacking 30 its CARD domain did not induce the luciferase reporter gene and pCI-CARD4LCARD $\Delta$ Only expressing the CARD-4 CARD domain did induce the luciferase reporter gene. Also, the CARD-4 LRR domains are not required for NF- $\kappa$ B pathway activation because pCI-CARD4LnoLRR expressing a CARD-4 35 mutant protein lacking LRR domains is able to induce the

- 128 -

luciferase reporter gene. In addition, the CARD-4 NBS domain is not sufficient for NF- $\kappa$ B pathway activation because pCI-CARD4NBSonly expressing CARD-4 NBS domain is not able to induce the luciferase reporter gene. In addition, the induction of the NF- $\kappa$ B pathway by CARD-4 is specific, as neither Apaf-expressing construct in this experiment induced luciferase activation.

In five transfection experiments, 293T cells coexpressing an NF- $\kappa$ B reporter plasmid (NF- $\kappa$ B-luciferase, Stratagene) and pCI-CARD-4L and either, no vector, pCI-TRAF6-DN (expressing a dominant negative version of TRAF-6), pCI-NIK-DN (expressing a dominant negative version of NIK kinase), pCI-CARD3CARDonly (expressing the CARD domain of CARD-3, which acts as a dominant negative version of CARD-3), or pCI-Bcl-XL (expressing the anti-apoptotic protein Bcl-XL) were created. TRAF6-DN, NIK-DN, and CARD3-CARDonly are dominant negative alleles of the TRAF6, NIK, and CARD3 genes, respectively. After 48 hours, cells were lysed and the relative luciferase activity was determined (Promega Kit) to assess NF- $\kappa$ B pathway activation by the genes expressed by the one or two pCI-based plasmids in each transfected cell line. The cell lines containing pCI-CARD-4L only or pCI-CARD-4L and pCI-Bcl-XL had relative luciferase reporter gene expression of about 18 units. The cell lines containing pCI-CARD-4L and pCI-TRAF6-DN, pCI-CARD-4L and pCI-NIK-DN, or pCI-CARD-4L and pCI-CARD3CARDonly had relative luciferase reporter gene expression of about 4 units. Inhibition of CARD-4L-mediated NF- $\kappa$ B pathway induction by TRAF6-DN, NIK-DN, and CARD-3CARDonly is specific as Bcl-XL did not inhibit CARD-4L-mediated NF- $\kappa$ B pathway induction.

These results demonstrate that dominant negative alleles of TRAF6, NIK and CARD-3 expressed, respectively, from pCI-TRAF6-DN, pCI-NIK-DN, and pCI-CARD3CARDonly



- 129 -

block induction of the NF- $\kappa$ B reporter gene by CARD-4L expression (pCI-CARD-4L) and suggest that TRAF6, NIK, and CARD-3 act downstream of CARD-4L to transduce the CARD-4L NF- $\kappa$ B pathway induction stimulus.

5           In an additional experiment, coexpression of CARD-4 and the CARD domain of RICK revealed that the CARD domain of RICK functions as a dominant negative mutant suggesting that RICK is a downstream mediator of CARD-4 function.

10 **Example 10:   Discovery of CARD-4 enhancement of caspase 9 activity.**

          In ten transfection experiments, 293T cells coexpressing a beta galactosidase-expressing plasmid (pCMV  $\beta$ -gal from Stratagene) as a marker for viable cells  
15 and either pCI, pCI-CARD-3, pCI-APAF, pCI-CARD-4L, pCI-CARD-4S, pCI-CARD4LnoLRR, pCI-CARD4NBSonly, pCI-CARD4LCARDonly, pCI-CARD-4LnoCARD or pCI-casp9 (expressing caspase-9) were created. Transfections included 400 ng of pCMV  $\beta$ -gal, 800 ng of expression  
20 plasmid, and Superfect transfection reagent from Qiagen and were carried out according to the manufacturer's directions. After 40-48 hours, cells were fixed and stained for beta-galactosidase expression and cell viability was determined by counting the number of beta  
25 galactosidase positive cells. Expression of pCI, pCI-CARD-3, pCI-APAF, pCI-CARD-4L, pCI-CARD-4S, pCI-CARD4LnoLRR, pCI-CARD4NBSonly, pCI-CARD4LCARDonly, and pCI-CARD-4LnoCARD did not result in loss of cell viability. As expected, expression of pCI-casp9 in 293T  
30 cells resulted in a loss of viability of about 75% of the cells in the experiment.

          It was next tested whether pCI, pCI-CARD-3, pCI-APAF, pCI-CARD-4L, pCI-CARD-4S, pCI-CARD4LnoLRR, pCI-CARD4NBSonly, pCI-CARD4LCARDonly, or pCI-CARD-4LnoCARD

- 130 -

would regulate caspase 9-mediated apoptosis. In nine transfection experiments, 293T cells coexpressing a beta galactosidase-expressing plasmid as a marker for viable cells, pCI-casp9, and either pCI, pCI-CARD-3, pCI-APAF, 5 pCI-CARD-4L, pCI-CARD-4S, pCI-CARD4LnoLRR, pCI-CARD4NBSoonly, pCI-CARD4LCARDonly, and pCI-CARD-4LnoCARD were created. After 40-48 hours, cells were fixed and stained for beta-galactosidase expression and cell viability was determined by counting the number of beta 10 galactosidase positive cells. Expression of pCI, pCI-CARD-4LnoCARD, and pCI-CARD4NBSoonly in the caspase 9-expressing 293T cells had no effect on the caspase 9-induced apoptosis. However, pCI-CARD-3, pCI-CARD-4L, pCI-CARD-4S, pCI-CARD4LnoLRR, pCI-CARD4LCARDonly and, as 15 expected, pCI-APAF enhanced the level of caspase 9-induced apoptosis to 20 or less beta galactosidase positive cells per experiment from about 100 beta galactosidase positive cells per experiment.

This experiment demonstrated that CARD-4 can 20 enhance caspase 9-mediated apoptosis because coexpression of CARD-4L or CARD-4S with caspase-9 dramatically increases caspase-9 mediated apoptosis. Furthermore, the CARD-4 CARD domain (SEQ ID NO:10) is necessary and sufficient for CARD-4-mediated enhancement of caspase-9- 25 potentiated apoptosis because CARD-4L lacking its CARD domain (pCI-CARD-4LnoCARD) does not enhance caspase-9-mediated apoptosis while the CARD-4 CARD domain expressed alone (pCI-CARD4LCARDonly) does induce caspase-9 mediated apoptosis. In addition, the LRR present in CARD-4 is not 30 required for CARD-4 enhancement of caspase-9-mediated apoptosis because expression of a CARD-4 protein lacking the LRR (pCI-CARD4LnoLRR) still enhances caspase-9-mediated apoptosis. The CARD-4 NBS is not sufficient for CARD-4 enhancement of caspase-9-mediated apoptosis 35 because expression of the CARD-4 NBS only (pCI-

- 131 -

CARD4NBSonly) does not enhance caspase-9 mediated apoptosis. This experiment also demonstrates that CARD-3 can enhance caspase-9-mediated apoptosis.

As detailed below in Example 12, CARD-4 does not  
5 appear to interact directly with caspase-9, suggesting that potentiation of caspase-9 activity by CARD-4 is mediated by activation of downstream pathways.

**Example 11: Identification and tissue distribution of mRNA species expressed by the human CARD-4 gene.**

10 Northern analysis of mRNAs extracted from adult human tissues revealed a 4.6 kilobase mRNA band that was expressed in most tissues examined. Highest expression was observed heart, spleen, placenta and lung. CARD-4 was also observed to be expressed in fetal brain, lung, liver  
15 and kidney. Cancer cell lines expressing the 4.6 kilobase CARD-4 mRNA include HeLa, K562, Molt4, SW480, A549 and melanoma. A larger 6.5 to 7.0 kilobase CARD-4 mRNA was expressed in heart, spleen, lung, fetal lung, fetal liver, and in the Molt4 and SW480 cell lines.

20 **Example 12: Physical association of CARD-4 with CARD-3.**

CARD-4-specific PCR primers with the 3' primer encoding the HA epitope tag were used to amplify the CARD-4L gene epitope tagged with HA and this PCR product was cloned into the mammalian expression vector pCI.  
25 CARD-3-specific PCR primers with the 5' primer encoding the MYC epitope tag were used to amplify the CARD-3 gene epitope tagged with MYC and this PCR product was cloned into the mammalian expression vector pCI. CARD-3-specific PCR primers with the 5' primer encoding the MYC epitope  
30 tag were used to amplify the CARD-3 gene lacking the CARD domain (SEQ ID NO:6) epitope tagged with MYC and this PCR product was cloned into the mammalian expression vector pCI. Caspase 9-specific PCR primers with the 3' primer

- 132 -

encoding the MYC epitope tag were used to amplify the caspase 9 gene epitope tagged with MYC and this PCR product was cloned into the mammalian expression vector pCI. In three transfection experiments, 293T cells  
5 coexpressing pCI-CARD-4LcHA and either pCI-CARD3nMYC, pCI-CARD3noCARDnMYC, or pCI-casp9cMYC were created. Cells from each transfected line were lysed and an immunoprecipitation procedure was carried out on each lysate with an anti-MYC epitope tag antibody to  
10 precipitate the CARD-4LcHA expressed by each cell line and any physically associated proteins. Immunoprecipitated proteins were separated by electrophoresis on denaturing polyacrylamide gels, transferred to nylon filters, and probed with an anti-HA  
15 epitope tag antibody in a Western blotting experiment to determine whether the MYC-tagged protein that was coexpressed with the CARD-4LcHA protein had coimmunoprecipitated with the CARD-4LcHA protein. In this experiment, CARD-3 was found to coimmunoprecipitate  
20 with CARD-4 while CARD-3 lacking its CARD domain and caspase-9 did not coimmunoprecipitate with CARD-4. This experiment demonstrates that CARD-4 and CARD-3 physically associate and that CARD-3 requires its CARD domain to associate with CARD-4. In addition, CARD-4 appears to  
25 not associate with caspase-9.

#### **Example 13: CARD-4 Genomic Sequence**

Figure 18 is depicts the 32042 nucleotide genomic sequence of CARD-4. This sequence is based the CARD-4 cDNA sequence described above and a BAC sequence (DBEST  
30 Accession No. AC006027). The CARD-4 cDNA sequence described above was used to correct three errors in the BAC sequence, including one error resulting in a frameshift. The CARD-4 genomic sequence of Figure 18 includes the following introns and exons: exon 1:

- 133 -

nucleotides 364-685, encoding amino acids 1-67 (start codon at nucleotides 485-487); intron 1: nucleotides 686-2094; exon 2: nucleotides 2095-2269, encoding amino acids 67-126; intron 2: nucleotides 2270-4365; exon 3: 5 nucleotides 366-6190, encoding amino acids 126-734; intron 3: nucleotides 6191-9024; exon 4: nucleotides 9025-9108, encoding amino acids 734-762; intron 4: nucleotides 9109-10355; exon 5: nucleotides 10356-10439, encoding amino acids 762-790; intron 5: nucleotides 10 10440-11181; exon 6: nucleotides 1182-11265, encoding amino acids 790-818; intron 6: nucleotides 11266-19749; exon 7: nucleotides 19750-19833, encoding amino acids 818-846; intron 7: nucleotides 19834-21324; exon 8: nucleotides 21325-21408, encoding amino acids 846-874; 15 intron 8: nucleotides 21409-24226; exon 9: nucleotides 24227-24310, amino acids 874-903; intron 9: nucleotides 24311-27948; exon 10: nucleotides 27949-28032, amino acids 903-930; intron 10: nucleotides 28033-31695; exon 11: nucleotides 31696-32024, encoding amino acids 930-953 20 (stop codon at nucleotides 31766-31768).

The introns in the CARD-4 genomic sequence contain consensus splice donor and acceptor sites (Molecular Cell Biology, Darnell et al., eds., 1996). The CARD-4 genomic sequence is useful for genetic identification and mapping 25 and identifying mutations, e.g., mutations is splice donor or splice acceptor sites.

### Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine 30 experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

- 134 -

What is claimed is:

1. An isolated nucleic acid molecule selected from the group consisting of:

- a) a nucleic acid comprising the nucleotide  
5 sequence of SEQ ID NO:38 or a complement thereof;
- b) a nucleic acid comprising the nucleotide  
sequence of SEQ ID NO:40 or a complement thereof;
- c) a nucleic acid comprising the nucleotide  
sequence of SEQ ID NO:42, or a complement thereof;
- 10 d) a nucleic acid molecule which encodes a  
polypeptide comprising the amino acid sequence of SEQ ID  
NO:38;
- e) a nucleic acid molecule which encodes a  
polypeptide comprising the amino acid sequence of SEQ ID  
15 NO:40;
- f) a nucleic acid molecule which encodes a  
polypeptide comprising the amino acid sequence of SEQ ID  
NO:42;
- g) a nucleic acid comprising the nucleotide  
20 sequence of SEQ ID NO:38 or a complement thereof;
- h) a nucleic acid consisting of the nucleotide  
sequence of SEQ ID NO:40 or a complement thereof;
- i) a nucleic acid consisting of the nucleotide  
sequence of SEQ ID NO:42, or a complement thereof;
- 25 j) a nucleic acid molecule which encodes a  
polypeptide consisting of the amino acid sequence of SEQ  
ID NO:38;
- k) a nucleic acid molecule which encodes a  
polypeptide consisting of the amino acid sequence of SEQ  
30 ID NO:40; and
- l) a nucleic acid molecule which encodes a  
polypeptide consisting of the amino acid sequence of SEQ  
ID NO:42.

- 135 -

2. A host cell which contains one of the nucleic acid molecules of claim 1.

3. An isolated polypeptide selected from the group consisting of:

- 5 a) a polypeptide comprising the amino acid sequence of SEQ ID NO:39;
- b) a polypeptide comprising the amino acid sequence of SEQ ID NO:41;
- c) a polypeptide comprising the amino acid  
10 sequence of SEQ ID NO:43;
- d) a polypeptide consisting of the amino acid sequence of SEQ ID NO:39;
- e) a polypeptide consisting of the amino acid sequence of SEQ ID NO:41;
- 15 f) a polypeptide consisting of the amino acid sequence of SEQ ID NO:43;
- g) a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO:39, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID  
20 NO:39;
- h) a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO:41, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:41;
- 25 i) a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO:43, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:43;
- 30 j) a naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence of SEQ ID NO:39, wherein the polypeptide is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule consisting of SEQ ID NO:38 under stringent conditions;

- 136 -

k) a naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence of SEQ ID NO:41, wherein the polypeptide is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule  
5 consisting of SEQ ID NO:40 under stringent conditions;  
and

l) a naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence of SEQ ID NO:43, wherein the polypeptide is encoded by a nucleic  
10 acid molecule which hybridizes to a nucleic acid molecule consisting of SEQ ID NO:42 under stringent conditions.

4. An antibody which selectively binds to any one of the polypeptides of claim 3.

5. A method for producing a polypeptide selected  
15 from the group consisting of:

- a) a polypeptide comprising the amino acid sequence of SEQ ID NO:39;
- b) a polypeptide comprising the amino acid sequence of SEQ ID NO:41;
- 20 c) a polypeptide comprising the amino acid sequence of SEQ ID NO:43;
- d) a polypeptide consisting of the amino acid sequence of SEQ ID NO:39;
- e) a polypeptide consisting of the amino acid  
25 sequence of SEQ ID NO:41;
- f) a polypeptide consisting of the amino acid sequence of SEQ ID NO:43;
- g) a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO:39, wherein the fragment  
30 comprises at least 15 contiguous amino acids of SEQ ID NO:39;
- h) a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO:41, wherein the fragment



- 137 -

comprises at least 15 contiguous amino acids of SEQ ID NO:41;

i) a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO:43, wherein the fragment  
5 comprises at least 15 contiguous amino acids of SEQ ID NO:43;

j) a naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence of SEQ ID NO:39, wherein the polypeptide is encoded by a nucleic  
10 acid molecule which hybridizes to a nucleic acid molecule consisting of SEQ ID NO:38 under stringent conditions;

k) a naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence of SEQ ID NO:41, wherein the polypeptide is encoded by a nucleic  
15 acid molecule which hybridizes to a nucleic acid molecule consisting of SEQ ID NO:40 under stringent conditions;  
and

l) a naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence of SEQ ID NO:43, wherein the polypeptide is encoded by a nucleic  
20 acid molecule which hybridizes to a nucleic acid molecule consisting of SEQ ID NO:42 under stringent conditions;

comprising the step of culturing the host cell of claim 2 under conditions in which the nucleic acid  
25 molecule is expressed.

6. A method for detecting the presence of a polypeptide of claim 2 in a sample, comprising:

a) contacting the sample with a compound which selectively binds to a polypeptide of claim 2; and  
30 b) determining whether the compound binds to the polypeptide of claim 2 in the sample.

- 138 -

7. A method for detecting the presence of a nucleic acid molecule of claim 1 in a sample, comprising the steps of:

a) contacting the sample with a nucleic acid probe  
5 or primer which selectively hybridizes to the nucleic acid molecule; and

b) determining whether the nucleic acid probe or primer binds to a nucleic acid molecule in the sample.

8. A method for identifying a compound which  
10 binds to a polypeptide of claim 2 comprising the steps of:

a) contacting a polypeptide, or a cell  
expressing a polypeptide of claim 2 with a test compound;  
and

15 b) determining whether the polypeptide binds to the test compound.

9. The method of claim 8, wherein the binding of the test compound to the polypeptide is detected by a method selected from the group consisting of:

20 a) detection of binding by direct detecting of test compound/polypeptide binding;

b) detection of binding using a competition binding assay; and

c) detection of binding using an assay for  
25 CARD-4L or CARD-4S mediated signal transduction.

10. A method for modulating the activity of a polypeptide of claim 2 comprising contacting a polypeptide or a cell expressing a polypeptide of claim 2 with a compound which binds to the polypeptide in a  
30 sufficient concentration to modulate the activity of the polypeptide.

- 139 -

11. A method for identifying a compound which modulates the activity of a polypeptide of claim 2, comprising:

- a) contacting a polypeptide of claim 2 with a test compound; and
- b) determining the effect of the test compound on the activity of the polypeptide to thereby identify a compound which modulates the activity of the polypeptide.

12. A method for identifying a compound that blocks the interaction between a CARD-4 protein comprising a CARD-4 domain and a CARD-4-interacting protein comprising the steps of:

- a) incubating said CARD-4 protein and said interactor in the presence and absence of a test agent;
- b) determining whether said test agent reduces the binding of said CARD-4 protein and said interactor; and
- c) identifying a compound that blocks the interaction of said CARD-4 protein with said interactor when said compound reduces the binding of said CARD-4 protein with said interactor;

wherein said interactor is selected from the group consisting of CARD-3 and hNUDC and wherein said CARD-4 domain comprises amino acids 1-145 of an amino acid sequence selected from the group consisting of SEQ ID NO:8 and SEQ ID NO:43.

13. The method of claim 12, wherein the CARD-4 protein comprising a CARD-4 domain is selected from the group consisting of:

- a) a polypeptide comprising the amino acid sequence of SEQ ID NO:8;
- b) a polypeptide comprising the amino acid sequence of SEQ ID NO:39;

- 140 -

c) a polypeptide comprising the amino acid sequence of SEQ ID NO:41; and

d) a polypeptide comprising the amino acid sequence of SEQ ID NO:43.

5           14. The method of claim 12, wherein the CARD-4 protein and interactor are expressed in a recombinant prokaryotic or eukaryotic cell line or wherein the CARD-4 protein and interactor are isolated proteins or present in cell-free protein extracts.

10           15. A method for identifying a compound that inhibits the induction of the NF- $\kappa$ B pathway by a CARD-4 protein comprising the steps of:

a) incubating a recombinant cell line containing a vector expressing CARD-4 in the presence and absence of  
15 a test agent;

b) determining whether said test agent inhibits the induction of the NF- $\kappa$ B pathway by CARD-4; and

c) identifying a compound that inhibits the induction of the NF- $\kappa$ B pathway by CARD-4.

20           16. The method of claim 15, wherein the CARD-4 protein comprising a CARD-4 domain is selected from the group consisting of:

a) a polypeptide comprising the amino acid sequence of SEQ ID NO:8;

25           b) a polypeptide comprising the amino acid sequence of SEQ ID NO:39;

c) a polypeptide comprising the amino acid sequence of SEQ ID NO:41; and

30           d) a polypeptide comprising the amino acid sequence of SEQ ID NO:43.

- 141 -

17. The method of claim 15 further comprising:

- a) incubating a recombinant cell line expressing CARD-4 and also expressing an NF- $\kappa$ B pathway reporter gene in the presence and absence of a test agent;
- 5 b) determining whether said test agent inhibits the induction of the NF- $\kappa$ B pathway reporter gene by CARD-4; and
- c) identifying a compound that inhibits the induction of the NF- $\kappa$ B pathway reporter gene by CARD-4.

10 18. A method for identifying a compound that inhibits the enhancement of caspase 9 activity by a CARD-4 protein comprising a CARD-4 domain comprising the steps of:

- a) incubating a recombinant cell line expressing caspase 9 and CARD-4 in the presence and absence of a test agent;
- 15 b) determining whether said test agent inhibits caspase 9 activity; and
- c) identifying a compound that inhibits the enhancement of caspase 9 activity by a CARD-4 protein.

19. The method of claim 18 further comprising:

- a) incubating a recombinant cell line expressing caspase 9 and CARD-4 and a beta-galactosidase expression vector in the presence and absence of a test agent;
- 25 b) determining whether the presence of said test agent increases the proportion of cells that stain positive for beta-galactosidase; and
- c) identifying a compound that inhibits the enhancement of caspase 9 activity by a CARD-4 protein by
- 30 identifying a compound that increases the proportion of cells that stain positive for beta-galactosidase.

- 142 -

20. The method of claim 18, wherein the CARD-4 protein comprising a CARD-4 domain is selected from the group consisting of:

- a) a polypeptide comprising the amino acid  
5 sequence of SEQ ID NO:8;
- b) a polypeptide comprising the amino acid sequence of SEQ ID NO:39;
- c) a polypeptide comprising the amino acid sequence of SEQ ID NO:41; and
- 10 d) a polypeptide comprising the amino acid sequence of SEQ ID NO:43.

21. A method for identifying a compound that inhibits the enhancement of caspase 9 activity by a CARD-3 protein comprising a CARD-3 domain comprising the  
15 steps of:

- a) incubating a recombinant cell line expressing caspase 9 and CARD-3 in the presence and absence of a test agent;
- b) determining whether said test agent inhibits  
20 caspase 9 activity; and
- c) identifying a compound that inhibits the enhancement of caspase 9 activity by a CARD-3 protein.

22. The method of claim 21 further comprising:

- a) incubating a recombinant cell line expressing  
25 caspase 9 and CARD-3 and a beta-galactosidase expression vector in the presence and absence of a test agent;
- b) determining whether the presence of said test agent increases the proportion of cells that stain positive for beta-galactosidase; and

- 143 -

c) identifying a compound that inhibits the enhancement of caspase 9 activity by a CARD-3 protein by identifying a compound that increases the proportion of cells that stain positive for beta-galactosidase.

CCACGCGTCCGGTCAGCTCTGGTTCCGAGAAGCAGCGGGCTGGCGTGGGCCATCCGGGGAATGGGC  
GCCCTCGTGACCTAGTGTTGCGGGGCAAAAAGGGTCTTSCCGGCCTCGCTCGTGACAGGGGCGTAT  
TTGGGCGCCTGAGCGCGGCGTGGGAGCCTTGGGAGCCGCGCCAGCAGGGGGCACACCCGGAACCG  
GCCTGAGCGCCCCGGGACCATGAACGGGGGAGGCCATCTGCAGCGCCCTGCCACCATTCCTTACCA  
CAAACTCGCGGACCTGCGCTACCTGAGCCGCGGCGCCTCTGGCACTGTGTCTCGTCCGCCCCGCCAG  
CAGACTGGCGCGTCCAGGTGGCCGTGAAGCACCTGCACATCCACACTCCGCTGCTCGACAGTGAA  
AGAAAGGATGTCTTAAGAGAAGCTCAAATTTTACACAAAGCTAGATTTAGTTACATTCTTCCAAT  
TTTGGGAATTTGCAATGAGCCTCAATTTTGGGAATAGTTACTGAATACATGCCAAATCGATCAT  
TAAATGAACTCCTACATAGGAAAACCTGAATATCCTGATGTTGCTTGGCCATTGAGATTTCTCTATC  
CTGCATGAAATTGCCCTTGGTGTAATTTACCTGCACAATATGACTCCTCCTTTACTTCATCATGA  
CTTGAAGACTCAGAATATCTTATTGGACAATGAATTTTATGTTAAGATTGCAGATTTTGGTTTAT  
CAAAGTGGCGCATGATGTCCCTCTCACAGTCACGAAGTAGCAAATCTGCACCAGAAGGAGGGACA  
ATTATCTATATGCCACCTGAAAACCTATGAACCTGGACAAAAATCAAGGGCCAGTATCAAGCACGA  
TATATATAGCTATGCAGTTATCACATGGGAAGTGTTATCCAGAAAAACAGCCTTTTGAAGATGTCA  
CCAATCCTTTGCAGATAATGTATAGTGTGTCAAGGACATCGACCTGTTATTAATGAAGAAAGT  
TTGCCATATGATATACCTCACCGAGCACGTATGATCTCTCTAATAGAAAGTGGATGGGCACAAAA  
TCCAGATGAAAGACCATCTTTCTTAAATGTTTTAATAGAACTTGAACCAGTTTTGAGAACATTTG  
AAGAGATAACTTTTTCTTGAAGCTGTTATTCAGCTAAAGAAAACAAAGTTACAGAGTGTTTCAAGT  
GCCATTCACCTATGTGACAAGAAGAAAATGGAATTATCTCTGAACATACCTGTAAATCATGGTCC  
ACAAGAGGAATCATGTGGATCCTCTCAGCTCCATGAAAATAGTGGTTCTCCTGAAACTTCAAGGT  
CCCTGCCAGCTCCTCAAGACAATGATTTTTTATCTAGAAAAGCTCAAGACTGTTATTTTATGAAG  
CTGCATCACTGTCTTGAAATCACAGTTGGGATAGCACCATTTCTGGATCTCAAAGGGCTGCATT  
CTGTGATCACAAAGACCATTCATGCTCTTCAGCAATAATAAATCCACTCTCAACTGCAGGAAACT  
CAGAACGTCTGCAGCCTGGTATAGCCAGCAGTGGATCCAGAGCAAAAGGGAAGACATTGTGAAC  
CAAATGACAGAAGCCTGCCTTAACCAAGTCGCTAGATGCCCTTCTGTCCAGGGACTTGATCATGAA  
AGAGGACTATGAACTTGTTAGTACCAAGCCTACAAGGACCTCAAAGTCAGACAATTACTAGACA  
CTACTGACATCCAAGGAGAAGAATTTGCCAAAGTTATAGTACAAAAATTGAAAGATAACAAACAA  
ATGGGTCTTCAGCCTTACCCGGAATACTTGTGGTTTCTAGATCACCATCTTTAAATTTACTTCA  
AAATAAAAGCATGTAAGTGACTGTTTTTCAAGAAGAAATGTGTTTCATAAAAGGATATTTATAAA  
AA (SEQ ID NO:1)

FIG. 1



Met	Asn	Gly	Glu	Ala	Ile	Cys	Ser	Ala	Leu	Pro	Thr	Ile	Pro	Tyr	His	Lys	Leu	Ala	Asp	22
Leu	Arg	Tyr	Leu	Ser	Arg	Gly	Ala	Ser	Gly	Thr	Val	Ser	Ser	Ala	Arg	His	Ala	Asp	42	
Arg	Val	Gln	Val	Ala	Val	Lys	His	Leu	His	Ile	His	Thr	Pro	Leu	Leu	Asp	Ser	Gln	Arg	60
Lys	Asp	Val	Leu	Arg	Glu	Ala	Glu	Ile	Leu	His	Lys	Ala	Arg	Phe	Ser	Tyr	Ile	Leu	Pro	80
Ile	Leu	Gly	Ile	Cys	Asn	Glu	Pro	Glu	Phe	Leu	Gly	Ile	Val	Thr	Glu	Tyr	Met	Pro	Asn	100
Gly	Ser	Leu	Asn	Glu	Leu	Leu	His	Arg	Lys	Thr	Glu	Tyr	Pro	Asp	Val	Ala	Trp	Pro	Leu	120
Arg	Phe	Arg	Ile	Leu	His	Glu	Ile	Ala	Leu	Gly	Val	Asn	Tyr	Leu	His	Asn	Met	Thr	Pro	140
Pro	Leu	Leu	His	His	Asp	Leu	Lys	Thr	Gln	Asn	Ile	Leu	Leu	Asp	Asn	Gln	Phe	His	Val	160
Lys	Ile	Ala	Asp	Phe	Gly	Leu	Ser	Lys	Trp	Arg	Met	Met	Ser	Leu	Ser	Gln	Ser	Arg	Ser	180
Ser	Lys	Ser	Ala	Pro	Glu	Gly	Gly	Thr	Ile	Ile	Tyr	Met	Pro	Pro	Glu	Asn	Tyr	Gln	Pro	200
Gly	Gln	Lys	Ser	Arg	Ala	Ser	Ile	Lys	His	Asp	Ile	Tyr	Ser	Tyr	Ala	Val	Ile	Thr	Trp	220
Glu	Val	Leu	Ser	Arg	Lys	Gln	Pro	Phe	Glu	Asp	Val	Thr	Asn	Pro	Leu	Gln	Ile	Met	Tyr	240
Ser	Val	Ser	Gln	Gly	His	Arg	Pro	Val	Ile	Asn	Glu	Glu	Ser	Leu	Pro	Tyr	Asp	Ile	Pro	260
His	Arg	Ala	Arg	Met	Ile	Ser	Leu	Ile	Glu	Ser	Gly	Trp	Ala	Gln	Asn	Pro	Asp	Gln	Arg	280
Pro	Ser	Phe	Leu	Lys	Cys	Leu	Ile	Glu	Leu	Glu	Pro	Val	Leu	Arg	Thr	Phe	Glu	Gln	Ile	300
Thr	Phe	Leu	Glu	Ala	Val	Ile	Gln	Leu	Lys	Lys	Thr	Lys	Leu	Gln	Ser	Val	Ser	Ser	Ala	320
Ile	His	Leu	Cys	Asp	Lys	Lys	Lys	Met	Glu	Leu	Ser	Leu	Asn	Ile	Pro	Val	Asn	His	Gly	340
Pro	Gln	Glu	Glu	Ser	Cys	Gly	Ser	Ser	Gln	Leu	His	Glu	Asn	Ser	Gly	Ser	Pro	Gln	Thr	360
Ser	Arg	Ser	Leu	Pro	Ala	Pro	Gln	Asp	Asn	Asp	Phe	Leu	Ser	Arg	Lys	Ala	Gln	Asp	Cys	380
Tyr	Phe	Met	Lys	Leu	His	His	Cys	Pro	Gly	Asn	His	Ser	Trp	Asp	Ser	Thr	Ile	Ser	Gly	400
Ser	Gln	Arg	Ala	Ala	Phe	Cys	Asp	His	Lys	Thr	Ile	Pro	Cys	Ser	Ser	Ala	Ile	Ile	Asn	420
Pro	Leu	Ser	Thr	Ala	Gly	Asn	Ser	Glu	Arg	Leu	Gln	Pro	Gly	Ile	Ala	Gln	Gln	Trp	Ile	440
Gln	Ser	Lys	Arg	Glu	Asp	Ile	Val	Asn	Gln	Met	Thr	Glu	Ala	Cys	Leu	Asn	Gln	Ser	Leu	460
Asp	Ala	Leu	Leu	Ser	Arg	Asp	Leu	Ile	Met	Lys	Glu	Asp	Tyr	Glu	Leu	Val	Ser	Thr	Lys	480
Pro	Thr	Arg	Thr	Ser	Lys	Val	Arg	Gln	Leu	Leu	Asp	Thr	Thr	Asp	Ile	Gln	Gly	Glu	Glu	500
Phe	Ala	Lys	Val	Ile	Val	Gln	Lys	Leu	Lys	Asp	Asn	Lys	Gln	Met	Gly	Leu	Gln	Pro	Tyr	520
Pro	Glu	Ile	Leu	Val	Val	Ser	Arg	Ser	Pro	Ser	Leu	Asn	Leu	Leu	Gln	Asn	Lys	Ser	Met	540

(SEQ ID NO:2)

FIG. 2

TCCTCCCTTC CTCCCGTTCC AGTGCCTSCA GGGCAGTGGT CCGGCGCGGG AAGACCTCTT  
CAAGAACAAG GATCACTTCC AGTTCACCAA CCTCTTCCTG TCGGGGCTGT TGTCCAAAGC  
CAAAACAGAA CTCCTGCGGC ATCTGGTGCC CGCGGCAGCC CTGAGGAGAA AGCGCAAGGC  
CCTGTGGGCA CACCTGTTTT CCAGCCTGCG GGGCTACCTG AAGAGCCTGC CCCGCGTTCA  
GGTCGAAAGC TTCAACCAGG TGCAGGCCAT GCCCACGTTT ATCTGGATGC TGGGCTGCAT  
CTACGAGACA CAGAGCCAGA AGGTGGGGCA GCTGGCGGCC AGGGGCATCT GCGCCAACTA  
CCTCAAGCTG ACCTACTGCA ACGCCTGCTC GGCCGACTGC AGCGCCCTCT CCTTCGTCTT  
GCATCACTTC CCCAAGCGGC TGGCCCTAGA CCTAGACAAC AACAATCTCA ACGACTACGG  
CGTGCGGGAG CTGCAGCCCT GCTTCAGCCG CCTCACTGTT CTCAGACTCA GCGTAAACCA  
GATCACTGAC GGTGGGGTAA AGGTGCTAAG CGAAGAGCTG ACCAAATACA AAATTGTGAC  
CTATTTGGGT TTATACAACA ACCAGATCAC CGATGTCGGA GCCAGGTACG TCACCAAAT  
CCTGGATGAA TGCAAAGGCC TCACGCATCT TAACTGGGA AAAACAAAA TAACAAGTGA  
AGGAGGGAAG TATCTCGCCC TGGCTGTGAA GAACAGCAAA TCAATCTCTG AGGTTGGGAT  
GTGGGGCAAT CAAGTTGGGG ATGAAGGAGC AAAAGCCTTC GCAGAGGCTC TCGGGAACCA  
CCCCAGCTTG ACCACCCTGA GTCTTGCGTC CAACGGCATC TCCACAGAAG GAGGAAAGAG  
CCTTGCGAGG GCCCTGCAGC AGAACACGTC TCTAGAAATA CTGTGGCTGA CCCAAAATGA  
ACTCAACGAT GAAGTGGCAG AGAGTTTGGC AGAAATGTTG AAAGTCAACC AGACGTTAAA  
GCATTTATGG CTTATCCAGA ATCAGATCAC AGCTAAGGGG ACTGCCCAGC TGGCAGATGC  
GTTACAGAGC AACACTGGCA TAACAGAGAT TTGCCTAAAT GGAAACCTGA TAAACCAGA  
GGAGGCCAAA GTCTATGAAG ATGAGAAGCG GATTATCTGT TTCTGAGAGG ATGCTTTCCT  
BTTCATGGGG TTTTGTCCCT GGAGCCTCAG CAGCAAATGC CACTCTGGGC AGTCTTTTGT  
GTCAGTGTCT TAAAGGGGCC TCGCGAGGCG GGACTATCAG GAGTCCACTG CCTYCATGAT  
GCAAGCCAGC TTCCTGTGCA GAAGGTCTGG TCGGCAAACT CCCTAAGTAC CCGCTACAAT  
TCTGCAGAAA AAGAATGTGT CTTGCGAGCT GTTGTAGTTA CAGTAAATAC ACTGTGAAGA  
GAAAAA AAAA ACGGACGCGT GG (SEQ ID NO:7)

FIG. 3 (page 2 of 2)

TCCTCCCTTC CTCCCGTTCC AGTGCCTGCA GGGCAGTGGT CCGGCGCGGG AAGACCTCTT  
CAAGAACAAG GATCACTTCC AGTTCACCAA CCTCTTCCTG TCGGGGCTGT TGTCCAAAGC  
CAACAGAAA CTCCTGCGGC ATCTGGTGCC CGCGGCAGCC CTGAGGAGAA AGCGCAAGGC  
CCTGTGGGCA CACCTGTTTT CCAGCCTGCG GGGCTACCTG AAGAGCCTGC CCGCGTTCA  
BGTGAAAGC TTCAACCAGG TGCAGGCCAT GCCCAGTTC ATCTGGATGC TCGGTGTCAT  
CTACGAGACA CAGAGCCAGA AGGTGGGGCA GCTGGCGGCC AGGGGCATCT GCGCCAACTA  
CCTCAAGCTG ACCTACTGCA ACGCCTGCTC GGCCGACTGC AGCGCCCTCT CCTTCGTCCT  
GCATCACTTC CCCAAGCGGC TGGCCCTAGA CCTAGACAAC AACAATCTCA ACGACTACGG  
CGTGGCGGAG CTGCAGCCCT GCTTCAGCCG CCTCACTGTT CTCAGACTCA GCGTAAACCA  
GATCACTGAC GGTGGGGTAA AGGTGCTAAG CGAAGAGCTG ACCAAATACA AAATTGTGAC  
CTATTTGGGT TTATACAACA ACCAGATCAC CGATGTCGGA GCCAGGTACG TCACCAAAT  
CCTGGATGAA TGCAAAGGCC TCACGCATCT TAACTGGGA AAAACAAAA TAACAAGTGA  
AGGAGGGAAG TATCTCGCCC TGGCTGTGAA GAACAGCAA TCAATCTCTG AGGTTGGGAT  
GTGGGGCAAT CAAGTTGGGG ATGAAGGAGC AAAAGCCTTC GCAGAGGCTC TCGGGAACCA  
CCCCAGCTTG ACCACCCTGA GTCTTGCGTC CAACGGCATC TCCACAGAAG GAGGAAAGAG  
CCTTGCGAGG GCCCTGCAGC AGAACACGTC TCTAGAAATA CTGTGGCTGA CCCAAAATGA  
ACTCAACGAT GAAGTGGCAG AGAGTTTGGC AGAAATGTTG AAAGTCAACC AGACGTTAAA  
GCATTTATGG CTTATCCAGA ATCAGATCAC AGCTAAGGGG ACTGCCCAGC TGGCAGATGC  
GTTACAGAGC AACACTGGCA TAACAGAGAT TTGCCTAAAT GGAAACCTGA TAAAACCAGA  
GGAGGCCAAA GTCTATGAAG ATGAGAAGCG GATTATCTGT TTCTGAGAGG ATGCTTTTCT  
TTTCATGGGG TTTTGGCCCT GGAGCCTCAG CAGCAAATGC CACTCTGGGC AGTCTTTTGT  
GTCAGTGTCT TAAAGGGGCC TCGCAGGCG GGAATATCAG GAGTCCACTG CCTYCATGAT  
GCAAGCCAGC TTCCTGTGCA GAAGGTCTGG TCGGCAAACT CCCTAAGTAC CCGCTACAAT  
TCTGCAGAAA AAGAATGTGT CTTGCGAGCT GTTGTAGTTA CAGTAAATAC ACTGTGAAGA  
GAAAAAAAAA ACGGACGCGT GG (SEQ ID NO:7)

FIG. 3 (page 2 of 2)

MEEQGHSEMEIIPSESHPHIQLLKSNRELLVTHIRNTQCLVDNLLKNDYFSAEDAEIVCACPTQP  
DKVRKIIDLQVQSKGZEEVSEFFLYLLQQLADAYVDLRPWLLLEIGFSPSLLTQSKVVVNTDPVSRYT  
QQLRHHHLGRDSKFVLCYAQKEELLLEIYMDTIMELVGFSNESLGSLSLACLLEDHTTGILNEQG  
ETIFILGDAGVGKSMMLLQRLQSLWATGRLDAGVKFFFHFRCRMFSQFKESDRLCLQDLLFKHYCY  
PERDPEEVFAFLLRFPHVALFTFDGLDELHSDLDLSRVPDSSCPWEPAPHLVLLANLLSGKLLKG  
ASKLLTARTGIEVPROFLRKKVLLRGFSPSHLRAYARRMFPERALQDRLLSLEANPNLCSLCSV  
PLFCWIIFRCFQHFRAAFEGSPQLPDCTMTLTDVFLLVTEVHLNRMQPSLVQRNTRSPVETLHA  
GRDTLCSLGQVAHRGMEKSLFVFTQEEVQASGLQERDMQLGFLRALPELGPGGDQOSYEFFHLTL  
QAFFTAFFLVLDLDRVGTQELLRFFQEWMPAPAGAATTSCYFPFLPFQCLQGSGPAREDLFKNKDH  
QFTNLFLCGLLSKAKQKLLRHLVPAALRRKRKALWAHLFSSLRGYLKS LPRVQVESFNQVQAMP  
TFIWMLRCIYETQSQKVGQLAARGICANYLKLTYCNACSA DCSALS FVLHHFPKRLALDLDNNNL  
NDYGVRELQPCFSRLTVLRLSVNQITDGGVKVLSEELTKYKIVTYLGLYNNQITDVGARYVTKIL  
DECKGLTHLKLKGNKITSEGKYLALAVKNSKSISEVGMWGNQVGDEGAKAF AEALRNHPSLTTL  
SLASNGISTEGGKSLARALQQNTSLEILWLTQNELNDEVAESLAEMLKVNQTLKHLWLIONQITA  
KGTAQLADALQSNTGITEICLNGNLIKPEEAKVYEDEKRIICF (SEQ ID NO:8)

FIG. 4

CACGCGTCCGACTTGCTGAAGAATGACTACTTCTCGGCCGAAGATGCGGAGATTGTGT  
BTGCCTGCCCCACCCAGCCTGACAAGGTCCGCAAAATTCTGGACCTGGTACAGAGCAAG  
GGCGAGGAGGTGTCCGAGTTCTTCTCTACTTGCTCCAGCAACTCGCAGATGCCTACGT  
GGACCTCAGGCCTTGCTGCTGGAGATCGGCTTCTCCCCCTTCCCTGCTCACTCAGAGCA  
AAGTCGTGGTCAACACTGACCCAGTGAGCAGGTATACCCAGCAGCTGCGACACCATCTG  
GGCCGTGACTCCAAGTTCTGTGCTGTGCTATGCCCAGAAGGAGGAGCTGCTGCTGGAGGA  
GATCTACATGGACACCATCATGGAGCTGGTTGGCTTCAGCAATGAGAGCCTGGGCAGCC  
TGAACAGCCTGGCCTGCCTCCTGGACCACACCACCGGCATCCTCAATGAGCAGGGTGAG  
ACCATCTTCATCCTGGGTGATGCTGGGGTGGGCAAGTCCATGCTGCTACAGCGGCTGCA  
GAGCCTCTGGGCCACGGGCGGGCTAGACGCAGGGGTCAAATTCTTCTTCCACTTTCGCT  
GCCGCTAGTTTCACTGCTTCAAGGAAAGTGACAGGCTGTGTCTGCAGGACCTGCTCTTC  
AAGCACTACTGCTACCCAGAGCGGGACCCCCAGGAGGTGTTTGCCTTCTGCTGCGCTT  
CCCCACGTGGCCCTCTTACCTTCGATGGCCTGGACGAGCTGCACTCGGACTTGGACC  
TGAGCCGCGTGCCTGACAGCTCCTGCCCCCTGGGAGCCTGCCACCCCCCTGGTCTTGCTG  
GCCAACCTGCTCAGTGGGAAGCTGCTCAAGGGGGCTAGCAAGCTGCTCACAGCCCCGAC  
AGGCATCGAGGTCCCGCGCCAGTTCTGCGGAAGAAGGTGCTTCTCCGGGGCTTCTCCC  
CCAGCCACCTGCGCGCCTATGCCAGGAGGATGTTCCCCGAGCGGGCCCTGCAGGACCGC  
CTGCTGAGCCAGCTGGAGGCCAACCCCAACCTCTGCAGCCTGTGCTCTGTGCCCTCTT  
CTGCTGGATCATCTTCCGGTGCTTCCAGCACTTCCGTGCTGCCTTTGAAGGCTCACCAC  
AGCTGCCCCGACTGCACGATGACCCTGACAGATGTCTTCTCCTGGTCACTGAGGTCCAT  
CTGAACAGGATGCTAGCCCCAGCAGCCTGGTGACGCGGAACACACGCAGCCCAGTGGAGAC  
CCTCCACGCCCGCGGGACACTCTGTGCTCGCTGGGGCAGGTGGCCCCACCGGGCATGG  
AGAAGAGCCTCTTTGTCTTACCCAGGAGGAGGTGCAGGCCTCCGGGCTGCAGGAGAGA  
GACATGCAGCTGGGCTTCTGCGGGCTTTGCGGAGCTGGGCCCCGGGGGTGACCAGCA  
GTCCTATGAGTTTTTCCACCTCAGCCTCCTCACCTGTAAACTGGGATCCCAGTATAGA  
CTTTGGAAATCAGTAGACACCATATGCTTCAAAAAACAGGGGCTATTAAATGACATCA  
GGAGCCAGAAAGTCTCATGGCTGTGCTTTCTCTTGAAGTTTATACAACAACCAGATCAC  
CGATGTGCGAGCCAGACTGGGAAAAAACAAAATAACAAGTGAAGGAGGGAAGTATCTCG  
CCCTGGCTGTGAAGAACAGCAATCAATCTCTGAGGTGGGATGTGGGGCAATCAAGTT  
GGGATGAAGGAGCAAAAGCCTTCGAGAGGCTCTGCGGAACCACCCAGCTTGACCAC  
CCTGAGTCTTGCGTCCAACGGCATCTCCACAGAAGGAGGAAAGAGCCTTGCGAGGGCCC  
TGCAGCAGAAACGCTCTCTAGAAATACTGTGGCTGACCCAAAATGAACTCAACGATGAA  
BTGGCAGAGAGTTTGGCAGAAATGTTGAAAGTCAACCAGACGTTAAAGCATTATGGCT  
TATCCAGAATCAGATCACAGTCTTTTGTGTGCTGCTTAAAGGGGCTGCGCAGGCGG  
GACTATCAGGAGTCCACTGCCTCCATGATGCAAGCCAGCTTCTGTGCAGAAGGTCTGG  
TCGGCAAACTCCCTAAGTACCCGCTACAATTCTGCAGAAAAGAATGTGTCTTGCGAGC  
TGTTGTAGTTACAGTAAATACACTGTGAAGAGACTTTATTGCCTATTATAATTATTTT  
ATCTGAAGCTAGAGGAATAAAGCTGTGAGCAACAGAGGAGGCCAGCCTCACCTCATT  
CAACACCTGCCATAGGGACCAACGGGAGCGAGTTGGTCACCGCTCTTTTCATTGAAGAG  
TTGAGGATGTGGCACAAGTTGGTGCCAAGCTTCTTGAATAAAACGTGTTTGATGGATT  
AGTATTATACCTGAAATATTTCTTCTTCTCAGCACTTCCCATGTATTGATACTGGT  
CCCACTTCACAGCTGGAGACACCGGAGTATGTGCAGTGTGGGATTTGACTCCTCCAAGG  
TTTTGTGGAAAGTTAATGTCAAGGAAAGGATGCACCACGGGCTTTTAATTTTAATCCTG  
GAGTCTCACTGTCTGCTGGCAAAGATAGAGAATGCCCTCAGCTCTTAGCTGGTCTAAGA  
ATGACGATGCCTTCAAATGCTGCTTCCACTCAGGGCTTCTCCTCTGCTAGGCTACCT  
CCTCTAGAAGGCTGAGTACCATGGGCTACAGTGTCTGGCCTTGGGAAGAAGTGATTCTG  
TCCCTCCAAAGAAATAGGGCATGGCTTGCCCTGTGGCCCTGGCATCCAAATGGCTGCT  
TTGTCTCCCTTACCTCGTGAAGAGGGGAAGTCTCTTCTGCTCCCAAGCAGCTGAAG  
GGTGAATAAACGGGCGCCAAGACTCAGGGGATCGGCTGGGAACGGGCCAGCAGAGCAT  
GTTGGACACCCCCCACCATTGGTGGGCTTGTGGTGGCTGCTCCATGAGGGTGGGGGTGAT  
ACTATAAGACTCTGTCTCTTGGCAGCTCATTTGTTAATAAAATACTGAAAACACAA  
AA  
AAAAAAAAAAAAAA (SEQ ID NO:25)

FIG. 5  
6 / 35

HASDLLKNDYFSAEDAEIVCACFTQPDKVRKILDLVQSKGEEVSEFFLYLL  
QQLADAYVDLRPWLLLEIGFSPSLLTQSKVVVNTDPVSRYTQQLRHHLGRDS  
KFVLCYAQKEELLLEEIYMDTIMELVGFSNESLGSLSLACLLDHTTGILN  
EQGETIFILGDAGVGKSMLLQRLQSLWATGRLDAGVKFFFHFRCRMFSCFK  
ESDRCLCLQDLLFKHYCYPERDP EEVFAFLLRFPHVALFTFDGLDELHSDLD  
LSRVPDSSCPWEPAHPLVLLANLLSGKLLKGASKLLTARTGIEVPRQFLRK  
KVLLRGFSPSHLRAYARMFPERALQDRLLSQLEANPNLCSLCSVPLFCWI  
IFRCFQHFRAAFEGSPQLPDCTMTLTDVFLLVTEVHLNRMQPSSLVQRNTR  
SPVETLHAGRDTLCSLGQVAHRGMEKSLFVFTQEEVQASGLQERDMQLGFL  
RALPELGPGGDQQSYEFFHLSLLTCKTGIFV (SEQ ID NO:26)

FIG. 6

**FIG. 7**

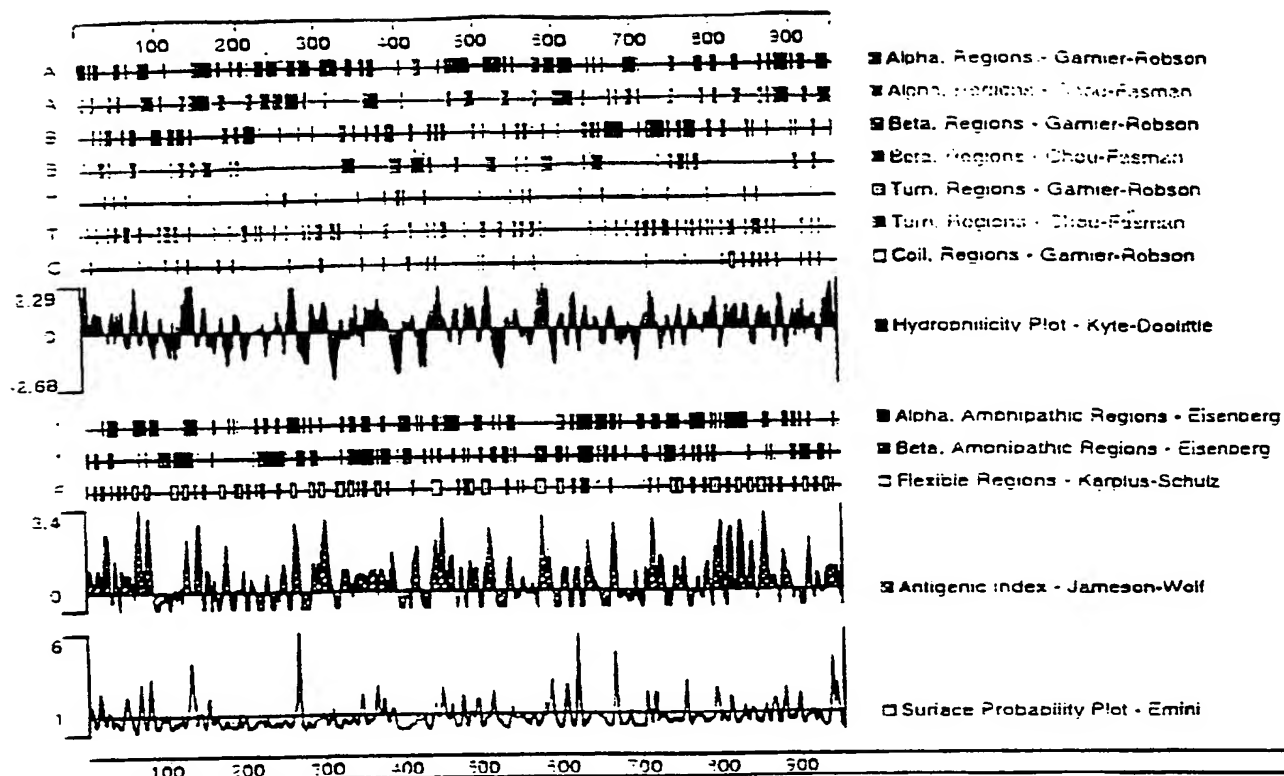


FIG. 8



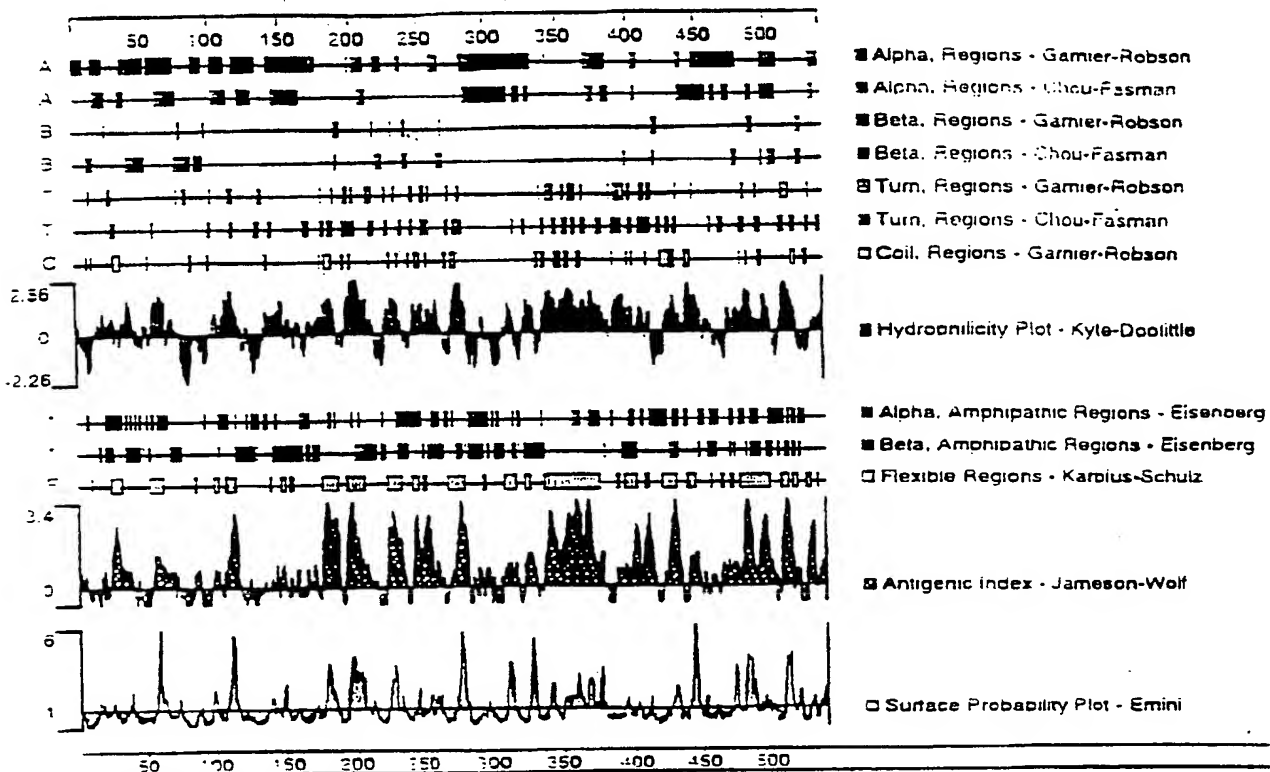


FIG. 9

CCCGCGTCCGCGTCCCCGGACCATGGCGCTCTCCGGGCTCTTCTCTAGCTCTCAGCGGCT  
GCGAAGTCTGTNAACCTGGTGGCCAAGTGATTGTAAGTCAGGAGACTTTCTTTCGGTTTC  
TGCCTTTGATGGCAAGAGGTGGAGATTGTGGCGGCGATTACAGAAAACATCTGGGAAGAC  
AAGTTGCTGTTTTTATGGGAATCGCAGGCTTGGAAGAGACAGAAGCAATTCAGAAATAA  
ATTGGAAATTGAAGATTTAAACAATGTTGTTTTAAATATTCTAACTTCAAAGAATGATG  
CCAGAACTTAAAAAGGGGCTGCGCAGAGTAGCAGGGGCCCTGGAGGGCGCGGCCTGAAT  
CCTGATTGCCCTTCTGCTGAGAGGACACACGCAGCTGAAGATGAATTTGGGAAAAGTAGC  
CGCTTGCTACTTTAACTATGGAAGAGCAGGGCCACAGTGAGATGGAAATAATCCCATCAG  
AGTCTCACCCCCACATTCAATTACTGAAAAGCAATCGGGAACCTTCTGGTCACTCACATCC  
GCAATACTCAGTGTCTGGTGGACAACTTGCTGAAGAATGACTACTTCTCGGCCGAAGATG  
CGGAGATTGTGTGTGCCTGCCCCACCCAGCCTGACAAGGTCCGCAAATTTCTGGACCTGG  
TACAGAGCAAGGGCGAGGAGGTGTCCGAGTTCTTCTCTACTTGCTCCAGCAACTCGCAG  
ATGCCTACGTGGACCTCAGGCCTTGGCTGCTGGAGATCGGCTTCTCCCCTTCCCTGCTCA  
CTCAGAGCAAAGTCGTGGTCAACACTGACCCAGTGAGCAGGTATACCCAGCAGCTGCGAC  
ACCATCTGGGCCGTGACTCCAAGTTCGTGCTGTGCTATGCCCAGAAGGAGGAGCTGCTGC  
TGGAGGAGATCTACATGGACACCATCATGGAGCTGGTTGGCTTCAGCAATGAGAGCCTGG  
GCAGCCTGAACAGCCTGGCCTGCCTCCTGGACCACACCACCGGCATCCTCAATGAGCAGG  
CTGCTTCAAGGAAAGTGACAGGCTGTGTCTGCAGGACCTGCTCTTCAAGCACTACTGCTA  
CCCAGAGCGGGACCCCGAGGAGGTGTTTGCTTCTGCTGCGCTTCCCCACGTGGCCCT  
CTTACCTTCGATGGCCTGGACGAGCTGCACTCGGACTTGAGCCTGAGCCGCGTGCCTGA  
CAGCTCCTGCCCCCTGGGAGCCTGCCCCACCCCTGGTCTTGCTGGCCAACCTGCTCAGTGG  
GAAGCTGCTCAAGGGGGCTAGCAAGCTGCTCACAGCCCGCACAGGCATCGAGGTCCCGCG  
CCAGTTCCTGCGGAAGAAGGTGCTTCTCCGGGGCTTCTCCCCCAGCCACCTGCGCGCCTA  
TGCCAGGAGGATGTTCCCCGAGCGGGCCCTGCAGGACCGCCTGCTGAGCCAGCTGGAGGC  
CAACCCCAACCTCTGCAGCCTGTGCTCTGTGCCCTCTTCTGCTGGATCATCTTCCGGTG  
CTTCCAGCACTTCCGTGCTGCCTTTGAAGGCTCACCACAGCTGCCCCGACTGCACGATGAC  
CCTGACAGATGTCTTCTCCTGGTCACTGAGGTCCATCTGAACAGGATGCAGCCAGCAG  
CCTGGTGCAGCGGAACACACGCAGCCAGTGAGACCTCCACGCCGCGCGGGACACTCT  
GTGCTCGCTGGGGCAGGTGGCCACCGGGGCATGGAGAAGAGCCTCTTTGTCTTACCCCA  
GGAGGAGGTGCAGGCCTCCGGGCTGCAGGAGAGAGACATGCAGCTGGGCTTCTGCGGGC  
TTTGCCCGAGCTGGGCCCCGGGGGTGACCAGCAGTCCTATGAGTTTTTCCACCTCACCT

FIG. 10 (Page 1 of 3)

FIG. 10 (Page 2 of 3)

GCTTTTAATTTTAATCCTGGAGTCTCACTGTCTGCTGGCAAAGATAGAGAATGCCCTCAG  
CTCTTAGCTGGTCTAAGAATGACGATGCCTTCAAAATGCTGCTTCCACTCAGGGCTTCTC  
CTCTGCTAGGCTACCCTCCTCTAGAAGGCTGAGTACCATGGGCTACAGTGTCTGGCCTTG  
GGAAGAAGTGATTCTGTCCCTCCAAAGAAATAGGGCATGGCTTGCCCCTGTGGCCCTGGC  
ATCCAAATGGCTGCTTTTGTCTCCCTTACCTCGTGAAGAGGGGAAGTCTCTTCCTGCCTC  
CCAAGCAGCTGAAGGGTGACTAAACGGGCGCCAAGACTCAGGGGATCGGCTGGGAACTGG  
GCCAGCAGAGCATGTTGGACACCCCCCACCATGGTGGGCTTGTGGTGGCTGCTCCATGAG  
GGTGGGGGTGATACTACTAGATCACTTGTCTCTTGCCAGCTCATTGTTAATAAAATAC  
TGAAAACCCAAAAAAAAAAAAAAAAAAAAAAAAAAGGGCGG (SEQ ID NO:38)

FIG. 10 (Page 3 of 3)

MEEQGHSEMEIIPSESHPHIQLLKSNRELLVTHIRNTQCLVDNLLKNDYFSAEDAEIVCA  
CPTQPDKVRKILDLVQSKGEEVSEFFLYLLOQLADAYVDLRPWLLEIGFSPSLLTQSKVV  
VNTDPVSRYTQQLRHHHLGRDSKFVLCYAQKEELLLEEIYMDTIMELVGFSNESLGSLNSL  
ACLLDHTTGILNEQAASRKVTGCVCRTCSSSTTATQSGTPRRCLPSCCASPTWPSSPSMA  
WTSCTRTWT (SEQ ID NO:39)

FIG. 11

CACGCGTCCGCGCTACTGCGGGAGCAGCGTCCTCCCGGGCCACGGCGCTTCCCGGCCCCG  
GCGTCCCCGGACCATGGCGCTCTCCGGGCTCTTCTCTAGCTCTCAGCGGCTGCGAAGTCT  
GTAAACCTGGTGGCCAAGTGATTGTAAGTCAGGAGACTTTCCTTCGGTTTCTGCCTTTGA  
TGGCAAGAGGTGGAGATTGTGGCGGCGATTACAGAAAACATCTGGGAAGACAAGTTGCTG  
TTTTTATGGGAATCGCAGGCTTGGAAGAGACAGAAGCAATTCCAGAAATAAATTGGAAAT  
TGAAGATTTAAACAATGTTGTTTTTAAATATTCTAACTTCAAAGAATGATGCCAGAACT  
TAAAAGGGGCTGCGCAGAGTAGCAGGGGCCCTGGAGGGCGCGGCCTGAATCCTGATTGC  
CCTTCTGCTGAGAGGACACACGCAGCTGAAGATGAATTTGGGAAAAGTAGCCGCTTGCTA  
CTTTAACTATGGAAGAGCAGGGCCACAGTGAGATGGAAATAATCCCATCAGAGTCTCACC  
CCCACATTCAATTACTGAAAAGCAATCGGGAACTTCTGGTCACTCACATCCGCAATACTC  
AGTGTCTGGTGGACAACCTTGCTGAAGAATGACTACTTCTCGGCCGAAGATGCGGAGATTG  
TGTGTGCCTGCCCCACCCAGCCTGACAAGGTCCGCAAATTTCTGGACCTGGTACAGAGCA  
AGGGCGAGGAGGTGTCCGAGTTCTTCCTCTACTTGCTCCAGCAACTCGCAGATGCCTACG  
TGGACCTCAGGCCTTGGCTGCTGGAGATCGGCTTCTCCCCTTCCCTGCTCACTCAGAGCA  
AAGTCGTGGTCAACACTGACCCAGGTAGGAGTCAGCCCCAGCAAGACCGCAGGCACCAGT  
GCAAGCAGGGCCCTGGGGGGTTTGGTAATGGCTGGGCCAGCCCTGAGTGCCACCTCAGGA  
AGCAGGCCCAGGTGCTATTTTGTATTTTAGAAAGGAACAGCTGAATCCTGTCTCCAAGTG  
CAGCCCAGGTGGCTGCGATTGAACTGCCCCACACCTCGATGGTCTGGTTTATAGAGGGGCC  
TTTGAAGTATGGGAATGGCCTGTGTTCTGACCCCTTGCTTTCTTCCTATTCTGACATAT  
GTAGACATTTTAAATGGTTGCACAAATTCAAGGTTGTATTTTTTTTTCTTTAAAAAATCT  
TTAGCTGGACATGGTAGCACACACCTGTAGTTCCAGCTACTCAGGAGGCTGAGGCAAGAG  
GACTGCTTGAGCCCCAGAGTCTAAGGCTGCAGCGAGCTATGATTGTGCCCCTACACTCCA  
CAGCCTGGGTTTTTAGAGTGAGACCCTGTCTCTAAAAAAAAAAAAAAAAAAAAAAAAAAAA  
AAAAAAAAAAAAANGGGCGG (SEQ ID NO:40)

FIG. 12

MEEQGHSEMEIIPSESHPHIQLLKSNRELLVTHIRNTQCLVDNLLKNDYFSAEDAEIVCA  
CPTQPDKVRKILDLVQSKGEEVSEFFLYLLOQLADAYVDLRPWLLEIGFSPSLLTQSKVV  
VNTDPGRSQPQQDRRHQCKQGPGGFGNGWASPECHLRKQAQVLF  
(SEQ ID NO:41)

FIG. 13

	M E E Q G H S E M E I I P S E S H P H I Q L L K S N R E L L V T H I R N T Q C L	Majority
	10 20 30 40	
	M E E Q G H S E M E I I P S E S H P H I Q L L K S N R E L L V T H I R N T Q C L	CARD4-Y CLONE
	M E E Q G H S E M E I I P S E S H P H I Q L L K S N R E L L V T H I R N T Q C L	CARD4-Z CLONE
	M E E Q G H S E M E I I P S E S H P H I O L L K S N R E L L V T H I R N T O C L	CARD4L
	V D N L L K N D Y F S A E D A E I V C A C P T Q P D K V R K I L D L V Q S K G E	Majority
	50 60 70 80	
1	V D N L L K N D Y F S A E D A E I V C A C P T Q P D K V R K I L D L V Q S K G E	CARD4-Y CLONE
1	V D N L L K N D Y F S A E D A E I V C A C P T Q P D K V R K I L D L V Q S K G E	CARD4-Z CLONE
1	V D N L L K N D Y F S A E D A E I V C A C P T Q P D K V R K I L D L V Q S K G E	CARD4L
	E V S E F F L Y L L Q Q L A D A Y V D L R P W L L E I G F S P S L L T Q S K V V	Majority
	90 100 110 120	
1	E V S E F F L Y L L Q Q L A D A Y V D L R P W L L E I G F S P S L L T Q S K V V	CARD4-Y CLONE
1	E V S E F F L Y L L Q Q L A D A Y V D L R P W L L E I G F S P S L L T Q S K V V	CARD4-Z CLONE
1	E V S E F F L Y L L Q Q L A D A Y V D L R P W L L E I G F S P S L L T O S K V V	CARD4L
	V N T D P V S R Y T Q Q L R H H L G R D S K F V L C Y A O K E E L L L E E I Y M	Majority
	130 140 150 160	
21	V N T D P V S R Y T Q Q L R H H L G R D S K F V L C Y A O K E E L L L E E I Y M	CARD4-Y CLONE
21	V N T D P G R S O P Q Q D R R H - - - - -	CARD4-Z CLONE
21	V N T D P V S R Y T Q Q L R H H L G R D S K F V L C Y A O K E E L L L E E I Y M	CARD4L
	D T I M E L V G F S N E S L G S L N S L A C L L D H T T G I L N E O X X X X - -	Majority
	170 180 190 200	
51	D T I M E L V G F S N E S L G S L N S L A C L L D H T T G I L N E O A A S R - -	CARD4-Y CLONE
37	- - - - -	CARD4-Z CLONE
51	D T I M E L V G F S N E S L G S L N S L A C L L D H T T G I L N E O G E T I F I	CARD4L
	- - - - - X C X X X X	Majority
	210 220 230 240	
39	- - - - - K V T G	CARD4-Y CLONE
37	- - - - - Q C K Q - -	CARD4-Z CLONE
31	L G D A G V G K S M L L Q R L Q S L W A T G R L D A G V K F F F H F R C R M F S	CARD4L
	C - - - - - X C - - - - -	Majority
	250 260 270 280	
33	C - - - - - V C - - - - -	CARD4-Y CLONE
41	- - - - -	CARD4-Z CLONE
41	C F H E S D R L C L Q D L L F K H Y C Y P E R D P E E V F A F L L R F P H V A L	CARD4L
	- - - - - X X C X X X - - - - -	Majority
	290 300 310 320	
36	- - - - - R T C S S S - - - - -	CARD4-Y CLONE
42	- - - - -	CARD4-Z CLONE
31	P T F D G L D E L H S D L D L S R V P D S S C P W E P A H P L V L L A N L L S G	CARD4L

FIGURE 14 (1 of 4)



-----TXXTXXXXP-----				Majority	
	330	340	350	360	
12	-----TTATQSGT <b>PR</b> -----				CARD4-Y CLONE
41	-----TTATQSGT <b>PR</b> -----				CARD4-Z CLONE
21	KLLKGASKLL <b>TAR</b> <b>T</b> GIEV <b>PR</b> QFLRKKVLLRGFSPSHLRAY				CARD4L
-----				RC Majority	
	370	380	390	400	
22	----- <b>RC</b> -----				CARD4-Y CLONE
41	----- <b>RC</b> -----				CARD4-Z CLONE
51	ARRMFPERALQDRLLSQLEANPNLCSLCSVPLFCWIT <b>FR</b> <b>RC</b>				CARD4L
-----LPXC-----				Majority	
	410	420	430	440	
24	----- <b>LPSC</b> -----				CARD4-Y CLONE
41	----- <b>LPSC</b> -----				CARD4-Z CLONE
51	FQHFRAAFEGSPQ <b>LPD</b> <b>C</b> XTMTLTDVFLLVTEVHLNRMQPS				CARD4L
-----				Majority	
	450	460	470	480	
28	-----				CARD4-Y CLONE
41	-----				CARD4-Z CLONE
51	LVQRNTRSPVETLHAGRDTLCSLGQVAHRGMEKSLFVFTQ				CARD4L
-----GPGG-----				Majority	
	490	500	510	520	
28	-----				CARD4-Y CLONE
41	----- <b>GPGG</b> -----				CARD4-Z CLONE
51	EEVQASGLQERDMQLGLRALPEL <b>GPGG</b> DQQSYEFFHLLTL				CARD4L
-----FXXXWXXP-----				CXX Majority	
	530	540	550	560	
28	----- <b>CAS</b> -----				CARD4-Y CLONE
45	----- <b>FGNGWASP</b> -----				CARD4-Z CLONE
51	CAFFTAFFLVLDDEVGTQELLR <b>FFQE</b> <b>WMP</b> <b>P</b> AGAATT <b>SCYP</b>				CARD4L
P-----				Majority	
	570	580	590	600	
51	<b>P</b> -----				CARD4-Y CLONE
53	-----				CARD4-Z CLONE
51	<b>P</b> FLPFQCLQGS GPAREDLFKNKDH FQFTNLF LCGLLSKAK				CARD4L
-----XWX-----				Majority	
	610	620	630	640	
52	----- <b>TWP</b> -----				CARD4-Y CLONE
53	-----				CARD4-Z CLONE
51	KELDRHLVPAALARRKKAL <b>W</b> AHLFSSSLRGY LKSLPRVQV				CARD4L

FIGURE 14 (2 of 4)

-----XXPXXXW-----				Majority
	650	660	670	680
3	-----SSPSMAW-----			CARD4-Y CLONE
3	-----SSPSMAW-----			CARD4-Z CLONE
1	ESFNQVQAMPPTFIWMLRCIYETQSQKVGQLAARGICANYL			CARD4L
-----XXCXXX-----				Majority
	690	700	710	720
2	-----TSGTRT-----			CARD4-Y CLONE
3	-----TSGTRT-----			CARD4-Z CLONE
1	ELTYCNAACSADCSALSFVLHHFPKRLALDLDNNNLNDYGV			CARD4L
-----				Majority
	730	740	750	760
8	-----			CARD4-Y CLONE
3	-----			CARD4-Z CLONE
1	RELQPCFSRLTVLRLSVYNQITDGGVKVLSEELTKYKIVTY			CARD4L
-----ECX-----				Majority
	770	780	790	800
8	-----			CARD4-Y CLONE
3	-----ECH-----			CARD4-Z CLONE
1	LGLYNNQITDVGARYVTKILD	ECKGLTHLSLYNNQITDVG		CARD4L
-----WXXXXXXXXXX-----				Majority
	810	820	830	840
3	-----WT-----			CARD4-Y CLONE
5	-----WT-----			CARD4-Z CLONE
1	ARLGKNKITSEGGKYLALAVKNSKSISEVGM	WGNQVGDEG		CARD4L
XXXXXXXXLXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX				Majority
	850	860	870	880
9	-----LRKQA-----			CARD4-Y CLONE
5	-----LRKQA-----			CARD4-Z CLONE
1	AKAFAEALRNHPSLTTLASNGISTEGGKSLARALQQNT			CARD4L
XXXXLXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX				Majority
	890	900	910	920
9	-----QVL-----			CARD4-Y CLONE
1	-----QVL-----			CARD4-Z CLONE
1	SLEILWLTONELNDEVAESLAEMLVNQTLKHLWLTIONQI			CARD4L
XX				Majority
	930	940	950	960
9	-----			CARD4-Y CLONE
1	-----			CARD4-Z CLONE
1	TAKGTAQLADALQSNTEITICLNGNLKPEEAKVYEDEK			CARD4L

FIGURE 14 (3 of 4)

---

<u>XXXXF</u>										Majority
19										
54	-	-	-	-	-	-	-	-	F	CARD4-Y CLONE
61	R	I	I	C	F					CARD4-Z CLONE
										CARD4L

ecoration (Decoration #1): Shade (with solid black) residues that match the Consensus exactly.

FIGURE 14 (4 of 4)

CCACGCGTCCGCGGACCGCGAGCGGTAGCGCCCTCCCTCCCAGCTGTTGTCCCGCCCGAT  
CCGCGACCCTAGTCCCCGGATCCCCTTGCTGAGAGTCACCGTACTCCAGGGCCAAGTGA  
CCAAAGTCCTGCCAACTTGGGTGAGCAATGAAAGGCAGGATCCTGGGTGGTGGCCCTGAA  
TCCTGATTTGTCTGCCCTGCCAGCGAGACACATGTGGTCAAAGATGAATTTGAGAAAAGT  
AGCTGCTGGCTACTTGAACAATGGAGGAACACGGCCATCATGAGATGGAAGGCACCCCAT  
TGGGTTGTCACTCCACATTAACTGCTGAAGATCAACAGGGAACATCTGGTCACCAACA  
TTCGGAACACTCAGTGTCTGGTGGACAACCTTGCTGGAGAATGGCTACTTCTCAGCCGAAG  
ATGCAGAGATTGTGTGTGCCTGTCCCACCAAGCCTGACAAGGTCCGAAAGATCCTTGACC  
TGGTGCAGAGCAAAGGCGAGGAGGTGTCTGAGTTCTTCCTCTACGTGCTGCAGCAGCTGG  
AGGATGCTTACGTGGACCTCAGGCTGTGGCTCTCAGAAATTGGCTTCTCCCCTTCCCAGC  
TCATTCCGACCAAACTATCGTCAATACTGACCCAGTAAGCAGGTATACCCAACAGCTGC  
GACACCAACTGGGCGCGACTCCAAGTTCATGCTGTGCTACGCCCAGAAGGAGGACCTGC  
TGCTGGAGGAGACCTATATGGACACACTCATGGGGCTGGTAGGCTTCAACAATGAAAACC  
TGGGCAGCCTAGGAGGCCTGGATTGCCTGCTGGACCACAGTACGGGCGTCCTCAACGAGC  
ATGGCGAGACTGTCTTCGTGTTTCGGGGACGCGGGAGTGGGCAAGTCCATGCTGCTGCAGA  
GGTTGCAGAGCCTCTGGGCGTCAGGCAGGTTGACCTCCACAGCCAAATTCTTCTTCCACT  
TCCGCTGCCGCATGTTTCAGCTGCTTCAAGGAGAGCGACATGCTGAGTCTGCAGGACCTGC  
TCTTCAAGCATTTCTGCTACCCGGAGCAGGACCCCGAGGAGGTGTTCTCCTTCTTGCTGC  
GCTTCCCCACACAGCGCTCTTCACTTTTGACGGCCTGGATGAGCTGCACTCAGACTTCG  
ACCTGAGCCGCGTGCCGGATAGCTGCTGCCCTTGGGAGCCGGCTCACCTCTGGTCTCTGC  
TGGCTAACCTCCTAAGTGGGAGGCTGCTCAAGGGTGCCGGCAAATTGCTCACTGCTCGCA  
CAGGCGTGGAGGTCCCCCGCCAGCTCCTGCGCAAAAAGGTGCTGCTCCGGGGCTTCTCCC  
CAAGTCACCTGCGCGCCTATGCCCGCCGGATGTTCCCCGAGCGCACAGCGCAGGAGCATC  
TGCTGCAGCAGCTGGATGCCAACCCCAACCTCTGCAGCCTGTGCGGGGTGCCGCTCTTCT  
GTTGGATCATCTTCCGTTGTTTCCAGCACTTCCAGACGGTCTTCGAGGGCTCCTCTTCAC  
AGTTGCCGACTGTGCTGTGACCCTGACCGATGTCTTTCTGCTGGTCACTGAGGTGCATC  
TGAACAGGCCGCGAGCCAGCAGCCTGGTGCAGCGCAACACGCGCAGCCCGGCGGAAACCC  
TACGTGCAGGCTGGCGCACGCTGCATGCGCTGGGAGAGGTGGCTCACCGAGGCACCGACA  
AGAGCCTCTTTGTGTTTGGCCAGGAGGAGGTGCAGGCGTCGAAGCTGCAGGAAGGAGATC  
TGCAGCTGGGCTTCTGCGGGCTTTGCCCGATGTGGGCCCTGAGCAGGGCCAGTCTTACG  
AATTTTTCCACCTTACGCTCCAGGCCTTCTTACCCGCTTCTTCTGGTAGCAGATGACA  
AAGTGAGCACCCGGGAGTTGCTGAGGTTCTTTCGAGAATGGACGTCTCCTGGAGAGGCAA

FIG. 15 (Page 1 of 3)

CAAGCTCGTCCTGCCATTCTTCCTTCTTCTCCTTCCAGTGCCTGGGCGGCAGAAGCCGGT  
TGGGCCCTGATCCTTTCAGGAACAAAGATCACTTCCAGTTCACCAACCTCTTCGTGTGCG  
GGCTACTGGCCAAAGCCCGACAGAACTCCTTCGGCAGCTGGTGCCCAAGGCTATCCTGA  
GGAGGAAGCGCAAGGCCCTGTGGGCTCACCTGTTTGCTAGCCTGCGCTCCTACTTGAAGA  
GCCTACCTCGGGTCCAGTCTGGAGGCTTTAACCAGGTGCATGCCATGCCACATTCTGT  
GGATGCTGCGCTGCATCTATGAGACGCAGAGCCAGAAGGTGGGGCGCCTCGCCGCCAGGG  
GCATCAGTGCGGACTACCTCAAGCTGGCCTTTTGCAACGCTTGCTCTGCGGACTGCAGCG  
CCCTGTCCTTCGTCTCTGCATCACTTCCACAGGCAGCTGGCCCTAGACCTGGACAACAACA  
ACCTCAATGACTATGGCGTGCAGGAGCTGCAGCCTTGCTTTAGCCGTCTCACGGTTATCA  
GACTCAGCGTCAACCAGATCACCGACACGGGGGTGAAGGTGCTATGTGAGGAACTGACCA  
AGTATAAGATCGTGACGTTCTTGGGTTTATACAACAACCAGATAACTGATATCGGAGCCA  
GGTATGTGGCCCAAATCCTGGATGAATGCAGAGGCCTCAAGCACCTTAAACTAGGGAAAA  
ACAGAATAACAAGTGAGGGCGGGAAGTGTGTGGCTTTGGCTGTGAAGAACAGCACCTCCA  
TCGTTGATGTTGGGATGTGGGGTAATCAGATTGGAGACGAAGGGGCAAAGGCCTTCGCAG  
AGGCATTGAAGGACCACCCAGCCTGACCACTCTCAGTCTTGCATTCAATGGCATCTCTC  
CGGAGGGAGGGGAAGAGCCTTGCGCAGGCCCTGAAGCAGAACACCACACTGACAGTAATCT  
GGCTGACCAAAAATGAACTTAATGATGAGTCTGCAGAGTGCTTCGCTGAGATGCTGAGAG  
TGAACCAGACGCTACGGCATTATGCTGATCCAGAATCGCATCACAGCCAAGGGGACAG  
CGCAGCTGGCGAGGGGCACTGCAGAAGAACACAGCCATAACAGAGATTTGTCTCAATGGAA  
ACTTGATTAAGCCCGAGGAGGCCAAAGTCTTCGAGAATGAGAAGAGAATCATCTGCTTCT  
GACGGACGCTCCTGGGCAGGATCTTTGTCCTAGGTTGCTCCTCAGTCACAGACAGCACTG  
TGCAGTCAGCAGGGTAGCAGGATGCTGTGCAGCGCCTGCAGCAAGGTGCCTGTGAGGAGC  
CCACACCTCCACAGTGCACACCGATGTCCCCTGCTCATGCTTGGAAGTGGTAGCACCCGCG  
CCGCGGCTGAGACCCTGCAGACGCAGGGAGTCTTAGGAACCATCGTCACCACTCAAAGCC  
AGCAGGGCATCTTCTGTACAAAGATCTCCCTGCATATCCACTAGACGGAAGCTGAAGGAA  
CGCAACAGCAGAGGAGGCCAACAGACGCCTGGCTGAAGGCTCCGTGGGACCAACGGTGTG  
ACCTTCAGAAAAGAGCTGGGAACTTGAGCAGAGCCGATGGTAACTTCTTGGGGAAAGAAG  
GCACCCAGTGACTGCATGGTTATTCTGAGTCCTCCTTCTCTGCTTAGTCCCTCTCACTG  
TACAGGTCTGTTTCTTCTCGCAGCTGTGGCTGCTGAAGTAGGTCCACTGTGGGGAGAGC  
TCATCACAGACTTTGGTTTCGGTTCTGGATTCTCAGTGGTGGCAACCGAGAGTCAGACGAT  
ACCCTCTAGGTGAGTCTCAGAGGATCTCTATGCTGTGAGAGGGTTGAGGGCCCAACCAGA  
ATTTTTTTTTTTTACCAGTTTTTTACTGTGCCTGCCCCAGGAGGGAGAATTACTTCCCAGC

FIG. 15 (Page 2 of 3)

CTCCACAGCAGCAGGCATGGCTTGCCTCAATGGTCCTGAGATCCCAACAAAACCTCTCTCC  
CTTGCCTGTGAGCAGAAAGTATCTTCATGTCCTCAGAAGTTGGAGGGTGACTGGACACAG  
TTAAGACTCAGAGAGCCAGCTGATAGCTCAAAGCAAAGCATGGCACATACCCACCACCAT  
ACCATGGTGCGCATGGGATGGGACAGTTGGAATGTTGCAGATAACGTGTTCTTTTGCCAG  
TTCATTTGTTAATAAAATATTTAAAACGTTAAAAAAAAAAAAAAAAAAAAAAAAAAGGGCG  
G (SEQ ID NO:42)

FIG. 15 (Page 3 of 3)

MEEHGHHEMEGTPLGCHSHIKLLKINREHLVTNIRNTQCLVDNLENGYFSAEDAEIVCA  
CPTKPKVRKILDLVQSKGEEVSEFFLYVLQQLEDAYVDLRLWLSEIGFSPSQLIRTKTI  
VNTDPVSRYTQQLRHQLGRDSKFMLCYAQKEDLLLEETYMDTLMGLVGFNNENLGS LGGL  
DCLLDHSTGVLNEHGETVFVFGDAGVGKSMMLLQRLQSLWASGRLTSTAKFFFHFRCRMFS  
CFKESDMLS LQDLLFKHFCYPEQDPEEVFSFLLRFPHTALFTFDGLDELHSDFDLSRVPD  
SCCPWEPAHPLVLLANLLSGRLLKGAGKLLTARTGVEVPRQLLRKKVLLRGFSPSHLRAY  
ARRMFPERTAQEHL LQQLDANPNLCSLCGVPLFCWII FRCFQHFQTVFEGSSS QLPDCAV  
TLTDVFLLVTEVHLNRPQPSSLVQRNTRSPAETLRAGWRTLHALGEVAHRGTDKSLFVFG  
QEEVQASKLQEGDLQLGFLRALPDVGPEQGQSYEFFHLLTQAFFTAFFLVADDKVSTREL  
LRFFREWTS PGEATSSSCHSSFFSFQCLGGRSRLGPD PFRNKDHFQFTNL FVCGLLAKAR  
QKLLRQLV PKAILRRKRKALWAHLFASLSYLKSLPRVQSGGFNQVHAMPTFLWMLRCIY  
ETQSQKVGRLAARGISADYLKLAFCNACSADCSALS FVLHHFHRQLALDLDNNNNLNDYGV  
QELQPCFSRLTVIRLSVNQITDTGVKVLCEELTKYKIVTFLGLYNNQITDIGARYVAQIL  
DECRGLKHLKLGKNRITSEGKGCVALAVKNSTSIVDVGMWGNQIGDEGAKAF AEALKDHP  
SLTTLSLAFNGISPEGKSLAQALKQNTTTLTVIWLTKNELNDESAECFAEMLRVNQTLRH  
LWLIQN RITAKGTAQLARALQKNTAITEICLNGNLIKPEEAKVFENEKRIICF  
(SEQ ID NO:43)

FIG. 16

25 / 35



FIG. 18 (1 of 10)

tgtatgattctgtttatatgaaatgtccagaaaaggtaaatctatagacaaaagcaaatacagtagt  
tgcctacggcccagggattggctacaaataggctccagaaaactctgggaagatggtagagatgt  
tctagacctggactgtggtgaggtttgcacaactttgtaaaacttactaaaaattactgacaaata  
tataacactccctaacactttgggagggccgaggtgggcagatcgcttgaacccaggaatttgaga  
ccagcctgggcaacatggcgagaccccgctctctacaaaaaacacaaaaattagtgtgggcttggg  
ggcatatgcctgtgtcccagctacttgggaggtgaggtgggaggattgcttgagcctgggaggt  
tgagactgcatgattgggtcactgcaccctagcctgagtgacagagcaggaccctatctctaaca  
acaaaaaagcagtggttgggtggaggagggccagcgtggccatctggcctggccctcgagtgcgagg  
ggcttcagtgttttagctgcagttcagtgatgacactgtgcggaggaataaagggtggcctgtctca  
gacactgatcccagctgaagtttgcaccttcttctggcaaatctgaggtcaagcagagagatc  
aaagcctggggccctcaggggtcaggaatgctggctctgtgacgctccccaggtcctgcacatctgag  
gagtggtgctgctggcctcagggcccaggttgtgaattttgtttatgcactcgccctctcctctt  
gagacctccctgtttgatgctgtttctgcctctctcctcaccctgctgctgtgcctgccacccc  
ctccctccagtgcagaggtatacccagcagctgcgacaccatctgggcccgtgactccaagttcgt  
gctgtgctatgccagaaggaggagctgctgctggaggagatctacatggacaccatcatggagc  
tggttggttcagcaatgagagcctgggcagcctgaacagcctggcctgctcctggaccacacc  
accggcatcctcaatgagcaggggtgagaccatcttcatcctgggtgatgctgggggtgggcaagtc  
catgctgctacagcgggtgcagagcctctgggcccacgggcccggctagacgcaggggtcaaattct  
tcttccactttcgtgcgcgcatgttcagctgcttcaaggaaagtgcagggctgtgtctgcaggac  
ctgctcttcaagcactactgctacccagagcgggaccccagaggaggtgtttgccttctctgctgcg  
cttccccacgtggccctcttccacttcgatggcctggacgagctgcactcggacttggacctga  
gcccgtgctgacagctcctgcccctgggagcctgcccaccccctgggtcttgcctggccaactg  
ctcagtgggaaagctgctcaagggggctagcaagctgctcacagcccgcacaggcatcgaggctcc  
gcgccagttcctgcggaagaaggtgcttctccggggcttctccccagccacctgcgcgctatg  
ccaggaggatgttccccgagcgggcccctgcaggaccgctgctgagccagctggaggccaacccc  
aacctctgcagcctgtgctctgtgcccctcttctgctggatcatcttccgggtgcttccagcactt  
ccgtgctgcctttgaaggctcaccacagctgcccgactgcacgatgaccctgacagatgtcttcc  
tcttggtcactgaggtccatctgaacaggatgcagcccagcagcctggtgcagcggaaacacacgc  
agcccagtgaggacccctccacgcggccgggacactctgtgctcgtggggcaggtggcccaccg  
gggcatggagaagagcctcttcttctcaccagaggaggtgcaggcctccgggctgcaggaga  
gagacactgcagctgggcttctgcggttctgcccagagctgggcccgggggagcagcagctcc  
tatgagtttttccacctcaccctccaggcctctttacagccttcttctcgtgctggacgacag  
ggtgggcaactcaggagctgctcaggttcttccaggagtgatgccccctgcgggggagcagacca  
cgtcctgctatcctcccttcttcccggttccagtgctgcagggcagtggtccggcgcggaagac  
ctcttcaagaacaaggatcacttccagttcaccaacctcttctgtgcgggctgttgtccaaagc  
caaacagaaaactccttcggcatctggtgcccgcggcagccctgaggagaaagcgcgaaggccctgt  
gggcacacctgttttccagcctgcggggctacctgaagagcctgccccgcgttcaggtcgaaagc  
ttcaaccaggtgcaggccatgccacgttcatctggatgctgcgctgcatctacgagacacagag  
ccagaaggtggggcagctggcgggccaggggcatctgcgccaactacctcaagctgacctactgca  
acgctgctcggccgactgcagcgcctctcctcgtcctgcacacttccccaaagcggctggcc  
ctagacctagacaacaacaatctcaacgactacggcgtgcgggagctgcagccctgcttcagccg  
cctcactgttctcaggtgaggctgccaggcaaggggagcaacaggtgggcccgggcccaggct  
cggaggggcatcggaatggcatcatggaccaggatccccaggactcatgaccatggcccttgga  
atgtccagaccttttcttcttagcagggcagaggtcaaggtgcaaagcttcgaggcaggtggac  
ctggatcagccacagctgggtgcccctgaacaaagtgttaactctcagagcctccacgcctca  
tctggaaaaagaagatgctcataatcctatcaattatggccacagggaccaatgttagttgagaa  
tgggtgaagtgcattacaaatattacctaattggaatgctctttacaacctgttaacttaggtact  
gttattgtctctattttggcagataaaggaagttagaggcacagagaagtttaatagcttgccttagg  
tcacacagctcagacatagcagtgccagaatgcataaagaaccttcccttttaagattaatgtaag  
gctccgagatagccctcaaaaagtcttctggaatatgggagcttttattactgcagagaaagcaga  
ccttgctgccagtgggactggtgacttctgtgatcaacgctagcagccctcacactgtagag  
acctcagttaaaatgctgactcgtggttgttttctgttccatagtttacgggaaacagagccca  
gtctgttttcttctattagcatttccatgtataaataaaccttgtaaatctctacaggggggttaa  
atttgccattacttgactcatgcatttctaaaaagcagtagggatttggaactgactcccagtg

FIG. 18 (2 of 10)

ctgtcacaccagtggtcagagtgtataaattgcatggggacatgggggtgcaggggggtcgaaggct  
gccctagcctgggaattggaaaacctggaggtctgttctctgtactctcagccagtgactctccct  
ctgtagccccaggcaggtctcacactcagtgccacctctgtccatcttttttttttctccccaa  
atggaggtcccgctctgttgcccaggctggagtgagtggtgatctcagctcactgcaacctcc  
gcctcctgggttcaagcgattctcttgcgccagcctcctgagtagctgggattacaggcacacgc  
caccatgtccggctaagttttttgtatttttagtaggacggggtttcccatgttggccaggctg  
gtcttgaaatcctgacctcaggtgatccgcccgcctcggccttccaaaatgttgggggttacaggc  
atgagccgcgcacccgacccctctgtccatcttttcaatgggaaactccacaccagtggtggtg  
ccctgcccttctgctgtccccagggtgaagctttccttcacaccagtgcaagaaaaaacagcttg  
taggaaagcagaggatattgggtaaccacgggaagcacactcagttctctgggtgcatcagttagg  
attagtttttagctgagagcgaaaaccccaaatgttgggtgagttacaagcttatttctctcatgta  
aaagtctagaggttaggtagttcaggactgggtatggaggtctccatgacctccggagcccaggctc  
tcttctgccttctgttctgcccactcactacccggctttcccatcttggcccaagagggtgc  
tcaaactccagccatctagtgcacactctagctatcagtaagaagggaagggaagattgagagc  
atgcctcaatcttttaagaacacttcttgggtattactaattatattgctgcttagatttcagaa  
cttaattgggtatgggcagaatttaattgagatgggcccagctaaaagatgggggaatctattgctaa  
gaaagtatagatattgggaatgtctagcagcctgtgctgtcttgggtggccatgccatgtacat  
acacactatttcccagcaccaagctggggactctgagggaaagggtccagagtgctctgacttgat  
cattttgatgtggcctaaaaatcaagcttttaattgttccagccttttacttggtatcaaggtcag  
cttgtgggtctaatgggcccagggttgtgtttctaaagtaagttttattgggaacgcagccata  
cccatttatttacttactgggtgcttcacactacacagttgagtagctgtgacagagaccacatg  
gccacagagcctaaaaatatttgcctgtctgacactttacagaatgacatgagcagctctccttga  
cagtgaggactcacagccttttccagtgacaaatcagggttagcccatgtgtttctggatgggggg  
aagctgttggcattttgggtataacagttcttgtgagacctgtccagcattttgcaggacacct  
acatcattggccctgctgcaagatgacagggcactccctcctccagtcacaaccactaaaagca  
gcccctgacatttccaaacccatgccctccaccatacagagaaccagggtacagggtctgggtgaca  
cataggtcacacgcaaagggtggatgtcagaggtgggtggcctcacacgtcctcctgtgtcctt  
cacggtcgtgtgaggagccagggggtgtgctgcagcctcgctcatgggctgggtgcaggatgggtc  
tggcgcccccacgttggccaggctttgttaaggggtctatttgggtgattgctgtggccattctcca  
ggggcgctctatacctgagaaaaactccagggcctgaaggcttctggatctttgttaagattaatgg  
ccttcataatgagtgccctgcctgactcgtaatttttttgcctgttttatttcagactcagcgtaa  
accagatcactgacggtggggttaagggtgctaagcgaagagctgaccaaatacaaaattgtgacc  
tatttgggggtatgtctttctccagaacactgggccaactacctagtaataatacagagctgcagg  
gaattcacattcccataggtccctggatgatcgccacggatgggcccagggtgggaagagcgctg  
gcccaggagttgagagtcctgggttctcttgggtcggccagtcaggaagtcttgcctgagcct  
cagcctcctcacctgtaaaactgggatccagctataggcaagtaggcttacaactgggtatttggg  
ggatgcaacgagaatataaggggatataatttaataaatgctagaatcctgtttacatattagtct  
ggactattttgggtccataatccctcatccagagcctttggggcaagacccgaatggggattctg  
agtgcctgctatggcatgacgtggccgcaggggtctaaaggcagtgccccattttcaaacactttc  
atatttctcccgcagaatgtatgaaacagtcacaaacagtggttaagaaagactataagtagct  
ccacatcagttgcaaaaagaattgtgagaaactttgggcattcagagcctttgagggttttggagt  
ctgagagaagggtatggggccagccccacacaactggtggctctgcaagctggagcagttgttc  
agtttcttggggcctcagtggtccttcgatgttaattgaggacatggacgcaaacgaccccgggcca  
cactcggtccagggtctgtgtgggtgtggaacctggaagcctgagcttagctgcctttcaac  
ttccatctgctgtactattgaattggcattgagcgggtgagatgggtgaaaggtagacatcgagaa  
gttttaataattcagaatcttttcttcaagacgctgaatgtaatcttagttgtaataaccatc  
acctgccagtcaccgagcactcatgcaccagggtttgcgttatgtcctaagatcctcataacca  
ccctgcaaggggactatcatcattacctctgtattacagatggagaaactgaggcacagagaggt  
aacgtgacttgtctcaggccataaagctggggaaagttagtgaggtgggttttgaacctgagctgt  
gagacctcagagccctaaactctggtgctgtgtgttcccctttcaaccagactttggaaatca  
gtagacaccatctgttcaaaaaacaggggctattaaaatgacatcaggagccagaaagtctcat  
ggctgtgctttcttgaagtttatacaacaaccagatcaccgatgtcgagccagggtacgtcac  
caaaatcctggatgaatgcaaaggcctcacgcactcttaagttaagtggggtaggcaccaggttcc  
tagtatattctcttgatcaccccttctgttgttcaaagattaaatgtcacagtaagagactttc

FIG. 18 (3 of 10)

atcctaaagccttccacttgtcccagggccatggttggtcaagtaaagatacctctgtgtgatctg  
tgaggcttggattcttggaggccctcccgttattggtaggggaaagggtggcatttttgatttca  
ttaactactagggccgaagaaaggactaactctcaccctttctgggtggtctttttgccccaaaggga  
gtttcctgtcggggttgcaaggaagagcttgggccccttgccctgctgtaggtgtgccctgcgcagg  
gggtgacagtgcgccaggcttggagcctctggctcctgccctgacagtggccacataccttgaccc  
ttggcagtc aaagtgggacctcccaggctctcccagggaagtcaagtgatgctgctgaggtcaatt  
agaggacccccaggagggtcaggctccctgagcttctgcagagactgtggaccatctcctggaga  
ggaacctgactgactgtcctcagggttcagttccctccctgacaggaggccaggccatggct  
cttgtggatcccagaagaaagtgtacggttcccaagatggggctggaaggggctctgtgctgggg  
aggagggtgacccacattggagcccctgcatagctggaggctgactgtgtgactctctctgca  
gactgggaaaaaaacaaaataacaagtgaaggagggaagtatctgcacctggctgtgaagaacagc  
aaatcaatctctgaggttgggtgagtagaaggggatggatgtatgtggtacaacctgctgtgtgt  
gtggggggcgggccttgtgttcttttcatacatcagtacaccagaaggaccactggggctcgct  
gtcggggagagatagtgagagcttccaccatgctgcgaaactgaaaccgtgcccatataagcaat  
aactccccggtccccctccccctgcctcttgcagccacctgctactactctctctatgggtt  
tgactactctacctcatgtgaagtggaaatcatacagatatttgcccttttggggatggctgatttca  
tagcatcatgtcctcaagattcgtccacatggaagcatgggacaggatttcccttttttttttt  
tttttttttttttgacagagtctcgctctgttgcccaggctggagtgcagtggcatgatctcg  
ctcactgcaacctctgccttctgggttcaagcgattctctgcctcagccacacgagtagctggg  
attataggcaccgcaccacatcccagctaatttttgatttttagtagaggcggggttccacca  
tgttggccaggctgggtctcaaaactcctgacctcaaatgatccaccacctcggtctcccaaagt  
tcaggattataggcgtgagccaccgtgcccgcaggatttccctcttttttaaggctgagtaat  
actccattgcatggctatgccacattttgtttactcattcatccaagaacagacactggcttgc  
tctatgctttggctgttgtgaataatgctgctgtgcacatgggcatacaaatgtctcttcaagga  
ctgccttcaattcttttttttttttttttttttttagattctttttttttttattataactcta  
agttttaggggtacatgtgcacattgtgacaggttagttacatatgtatacatgtgccatgctggg  
cgctgcacccactaatgtgtcatctagcattaggtatatctcccaatgctatccctccccctcc  
cccagccccaccacagtcaccagagtgtgatattcccttccctgtgtccatgtgatctcattgt  
caattcccacctatgagtgagaatatgcggtgtttgggtttttgttcttgcgatagtttactgag  
aatgatggtttccaatttcatccatgtccctacaaaggatatagaactcatcattttttatggctg  
catagtatccatgggtgtatatgtgccacattttcttaatccagtctatcattgttggacatttg  
gggtgggttccaagtctttgctattgtgaatagtgccacaataaacatacgtgtgcatgtgtctt  
atagcagcatgattttatactcatttgggtatataccagtaaatgggatggctgggtcaaatggta  
tttctagttctagatccctgaggaatcgccacactgacttccacaatgggttgaactagtttacag  
tcccaccaacagtgtaaaagtgttccatatttctccgcacctctccagcacctgctgttccctga  
ctttttaatgattgccatttctaactgggtgtgagatgatatactcatagtggttttgatttgcattt  
ctctgatggccagtgtgatgagcatttcttcatgtgttttttggtgcataaatgtcttctttt  
gagaagtgtctgttcatgtccttcgcccactttttgatgggggtgtttgttttttcttgtaaat  
ttgtttgagttcatgttagattctggatattagcccttgtcagatgagtaggttgcgaaaattt  
tctcccattgttaggtgcctgttcaactctgtaggttcttttcttctgtgtgcagaagctctt  
agttaaattagatcccatttgtcaattttgtcttttgttgccattgcttttgggtgttttggacat  
gaagtccttgcccacgcctatgtcctgaatggtaatgcctaggttttcttctagggtttttatgg  
ttttagggtttaacgtttaaatctttaatccatcttgaattgatttttgtataagggtgaaggaag  
ggatccagtttcagctttctacatatggctagccagtttcccagcaccattttatataatagga  
atcctttccccattgcttgttttctcagggttgtcaaagatcagatagttgtagatatgcggca  
ttatttctgagggtctgttctgttccattgatctatatctctgttttgggtaccagtaccatgct  
gttttgggttactgtagccttgtagtagtttgaagtcaagtagtgatgcctccagctttgtt  
cttttggcttaggattgacttggcgatgcgggtcttttttgggtccatatagaactttaagtag  
tttttccaattctgtgaagaaagtcatggtagcttgatggggatggcattgaatctgtaaat  
accttgggcagtagtgccttttccagatattgattcttccctacccatgagcattggaattgttctt  
ccatttgtttgttccctcttttatttcccttgagcagtggtttgtagttctccttgaagaggtcct  
tcacatcccttgaagttggattccctaggtattttattctcttgaagcaattgtgaatgggagt  
tcacccatgatttggctctctgttgtctgttgggtgtataagaatgcttgtgatttttgtac  
attgattttgtatcctgagacttgcctgaagttgcttatcagcttaaggagatttttgggctgaga

FIG. 18 (4 f 10)

cgatgggggttttctagataaacaatcatgtcgtctgcaaacagggacaatttgacttcctctttt  
cctaattgaataaccctttatttccctctcctgcctgattgccctggccagaacttccaacactat  
gttgaataggagcggtgagagagggcatccctgtcttgtgccagttttcaaagggaatgcttcca  
gtttttgcccattcagtatgatattggctgtgggtttgtcatagatagctcttatttttgaaa  
tacgtcccatcaatacctaattttattgagagtttttagcatgaagggttgttgaatttttgtcaaa  
ggctttttctgcatctattgagataatcatgtgggtttttgtctttggctctgtttatatgctgga  
ttacattttattgattttgcgtatattgaaccagccttgcatcccagggatgaagcccacttgatca  
tggtgggataagctttttgatgtgctgctggattcgggttgccagtatttttattgaggatttttgc  
atcaatgttcatcaaggatattgggtctaaaattctcttttttggttgtgtctctgcccggctttg  
gtatcagaatgatgctggcctcataaaatgagttaggaggattccctctttttctattgattgg  
aatagtttcagaaggaatggtaccagttcctccttgtagctctggtagaattcggctgtgaatcc  
atctggctcctggactcttttggttggttaaactattgattattgccacaatttcagagcctgtta  
ttggctctattcagagattcaacttctcctcctggttttagtcttgaggagagtgtatgtgtcgaggaat  
gtatccattttctctagattttctagttttatttgctgtagaggtgtttgtagattctctgatgg  
agtttgtattttctgtgggatcggtgggtgatatccctttatccattttttattgtgtctattgag  
tcttctctctttttttctttattagttctgtctagcgggtctatcaattttgttgatcctttcgaaa  
aaccagctcctggattcattgattttttgaagggtttttgtgtctctattttccttcagttctgc  
tctgatttttagttattttcttgcttctgtctagcttttgaaatgtgtttgtctcttgcttttctagt  
cttttaattgtgatgttaggggtgtcaattttggatctttcctgctttctcttgtaggcatttagt  
gctataaattttccctctacacactgctttgaatgcgtcccagagattctgggtatgtgggtgtctt  
gttctcgttggtttcaaagaacatctttattttctgccttcatttcgttatgtaccagtagtcat  
tcaggagcaggttggttcagtttccatgtagttgagcggctttgagtgagattcttaacccagtagt  
tctagtttgattgcactgtggtctgagagatagtttgttataaatttctgttcttttacatttgc  
gaggagagctttacttccaactatgtgggtcaattttggaatagggtgtgggtgtggtgctgaaaaa  
atgtatattctgttgatttgggggtggagagttctgtagatgtctattaggctctgcttggtgcaga  
gctgagttcaattcctgggtatccttggtgactttctgtctcattgatctgtctaatgttgacag  
tgggggtgttaaagtctccattatataatgtgtgggagtctaagtctctttgtaggctcactgagga  
cttgctttatgaatctgggtgctcctgtattgggtgcataaataatttaggatagttagctcctct  
tgttgaattgatccctttaccattatgtaatggccttctttgtctcttttgatctttgttggttt  
aaagtctgttttatcagagactaggattgcaaccctgcctttttttgttttccattggcttggt  
agatcttctccatcctttttattttgagcctatgtgtgtctctgcacgtgagatgggtttcctga  
atacagcacactgatgggtcttgactctttatccaacttgggtccatttctccacatcactttcagga  
atttagtccatttatatttaaagttaatattgtttatgtgtgaatttgatcctgtcattatgatgt  
tagctggcgattttgtcattagttgatgcagtttcttccctagttctcgatgggtctttacattttg  
gcatgattttgcagcggctgggtaccggtgttccctttccatgtttaccgcttccctcaggagctc  
ttttagggcaggcctgggtgggtgacaaaatctctcagcatttgcttgtctataaagtattttattt  
ctccttcacttatgaagcttagtttgggtggatataaaattctgggttgaaaattcttttcttta  
agaatgttgaatatatggccccccactctctctgggttgtaggggtttctgcccagagatccgctgt  
tagtctgatgggtttcctttgagggttaaccggaactttctctctggtgccttaacatttttt  
ccttcatttcaactttggtgaatctgacaattatgtgtcttgaggattgctcttctcgaggagtagt  
ctttgtggcgttctctgtatttctgaaatctgaacgttggcctgccttgctagattgggggaagtt  
ctcctggataaatatcctgcagagtggttttccaacttgggtccatttctccacatcactttcagga  
caccaatcagacgtagatttgggtcttttcacatagtcctcatatttcttgagggtttgctcattt  
ctttttattctttttctctaaacttcccttctcgcttcatttcatttcatttcatttcatttc  
tgataccctttcttccagttgatcgcatcggtcctgaggcttctgcattcttcacgtagttctc  
gagccttggttttcagctccatcagctcctttaagcacttctctgtattgggttattctagttata  
cattcttctaaaatttttttcaaagttttcaacttctttgcctttgggtttgaatgtcctccgtag  
ctcagagtaatttgatcgtctgaagccttcttctctcagctcgtcaaaaatcattctccatccagc  
tttgttctgttgctgggtgaggaactgcgttccctttggaggaggagagggcgtctgcgttttagag  
tttccagttttctgttctgttttttcccatctttgtgggtttatctacttttggtctttgatg  
atggtgatgtacagatgggttttcagtgtagatgtccttctgtggttggtagtttcttcttaaca  
gacaggacctcagctgcaggtctgttgggaataccctgcctggtgtgaggtgtcagtggtcctctgc  
tgggggggtgcctccagttaggctgctcgggggtcaggggtcagggaccacttgaggaggcag  
ctgcccgttctcagatctccagctgcgtgctggggagaaccactgctctcttcaaagctgtcagac

FIG. 18 (5 of 10)

agggacacttaagtctgcagaggttactgctgtctttttgtttgtctgtgcccctgccccagagg  
tggagcctacagaggcaggcaggtccttgagctgtggtgggctccaccagttcgagcttccc  
ggctgctttgtttacctaagcaagcctgggctatggcgggcgccccccccagcctcgttgccg  
ccttgacagtttgatctcagactgctgtgctagcaatcagcgagattccgtgggctgtaggacctc  
tgagccaggtgtgggatatagtctcgtggtgcgcctgttcttaagccggtctgaaaagcgcaata  
ttcgggtgggagtgacccgattttccaggtgcgtccgtcaccctttctttgactcggaaaggga  
actccctgatcccttgcgcttcccaggtgaggcaatgcctcgccctgcttcggctcgcgcacggg  
gcgcgcacacactggcctgcgcccactgtctggcgctccctagtgcagatgaaccgggtacctcag  
atggaaatgcagaaatcacccgtcttctgcgtcgctcacgctgggagctgtagaccggagctgtt  
cctattcggccatcttggtcctccctccaattctttgggtatatatccagcagtgaggattgtc  
ggatcacatggtaatttttaattttttgaagaatcatcatactgttttccacggcagcagacca  
ttttatgttcccaccaacagttcattctagtttctccacatccttgccaacacttgctattttct  
ctttttgacagtaacctcctaataatgagtggtgctgtctcattgtggttttgattcttgagg  
cttttttaagctttttgtttcattataattttttattggattacaaaagggaacacaggttaatttta  
tttgaaactatgaaaaataataaaaaattatcttctcagaaaatgattcttggttaacatttaagc  
tcagtttaagctctctcactttctctccctctctctctttgtacaacttttaaaaaatatagtag  
gggtgagactatatgtatctatactatagtaggggtgagactatatgtatccttcccttttctact  
taatctcatgccttgagtagctttccactttattaaaaatgtgatgccattcaattgtatagtaa  
atacatatatgtaagcaaaacactgaaaactcttattctgggttccagcaagccataacctggaat  
ggtgtaagcaggtagtttgcttggtgtgaacgtgttggtgaggcagctgccattgtgtgtgtagt  
gggccacacgaacttggtctgttggtgtgtagacagtggtgtgctgatcctattaggaacagccaac  
gctttgtgtgagccacacacgggttctaagtgtcttgcttctgttaactcagtgaaatcctcacaac  
tccatgacgggaatgtcttaattatccccattttatagatggggcaactgaggtccaagagactac  
ataatttcccgaaagttcacacaggttagcagatggcagagccgggtcaggagtcaccatcttacc  
acgcagactgttttagccagagactctccggatctgctgtaggggacagaatacagctttatcgc  
cgcacctgtccaccaagatggccgtagccacagagcttggttgggtaacgtcctctttatgtgac  
aggaacgttgctgatgggggttctgaaggtacttccctgctctttgtctcctggaagactgtgtct  
tcaggaatgtctctgacctgcccagagttgaacggatgctgggaaccacagcactgcacacggc  
cttccctccaggactctgcgcacctctgtgctccacaggagacatgcaggtgctttctctcatga  
gctcaggctcctgggtgacagctctccgaagctcgtggtgaggtcgggtctctaactgtgcccac  
ttgccgatggcctctgttcacaaggcttccctgctcttcgatcttgcatcacccttgaatttg  
aaatccagagcagccactcagagaccagtgtaggaattagtgtccaggccacagatccaggga  
ctgggcacaaacatctgcctgttgagttaggaactgagctgtggccattggcaaaaaaggaggggt  
gagcatggctgtttcttggggagctaacattcactatcttgctcctccctcaggatgtggggca  
atcaagttggggatgaaggagcaaaagccttcgcagaggtctgcggaaccaccccagcttgacc  
accctgaggttaactgtggccctgctgtctccaggggccaacctgggtccctcccagctgctctagg  
tttgctgggggaagggtgattcgtgctcctaataagaagaggaatttgcatgtgtgattttccttac  
tcttgctcaaacctttctttgatgcataagaggccatctagtaaagcacattcttctctttttta  
actttaagttctgggatacatgtagaagatgtgcaggtttgttacataggcaaatgcatgccatg  
gtgatttgctgcacctatcaacctgtcatctaggtttttaagccctgcatgcattaggatttgctc  
ctaattgcttgccttcccttgcctccaccccaacccaagccctgggtgtgtgtgtgttccctccat  
gtgtccatgtgttctcattgttcaactcccacttacgagtgagaacatgcagtggtttgggttttt  
gttccctgtgttagtttgctgagaatgatgggtttccagcttcatccatgtgccagcaaaaggacatg  
atctcatttttttttatgggtgcatagtattccatagtggtgtatgtgccacattttctttatcca  
gtctatcactgatgggcatttggttggttccaagcttttgctattgttaaatagtgtacaataa  
acatacatgtgcttggtgtctttatagcagaatgatttataatcctttgggtaaatacccagtaat  
gggattgtggtgcaaatggtatttctgggtctagatccctgaggaatcaccttaagtgtttatt  
cagctcagtgaaattctgcagtggtcccacaccagccaaccaccccccacaaagacagaggaca  
tttccagccctcagccatccctgcagtgctcccttgctggtagaggagggtttcctaagtgcaga  
tgaaacttaataagatgctggccagcagattcctgcccctccttgctcctcaggatgatgtgga  
aaagagggactcttctctctataaatggggatgcacctaccagcccccgcttaggtgctggtgc  
caaatctgggaccttggtatgtccacggctctgctgctgttcttctaccactgaaaaagagtc  
caagaagggtggggacagtagcagaagagactttgccaggtcttgcatggtgggtaccttgatggg  
gccagcctttagaaggacagcttgccaggcctcgccagcctcctgcccattgtgcagaaacctgag



gtgccgacccccagccccactgttggtgtgagcaggtgtgctgatgacccatttcccgtccagcctg  
cccttggtgctctgtgtgtggtgctctggggcagcagcgcctgggcactactgctgcagctgaacac  
ttctgcacccctgccccgagtgagcctgggctggggccacagccaggcagaggcttcccagctggt  
ctgatgttgaaagctaagattgaatgtagatgtgtctttaataattcaccccaagtggttccctc  
ctagtcttgcgctccaacggcatctccacagaaggaggaaagagccttgcgaggccctgagcag  
aacacgtctctagaaatactgtggtaatagtctcgagtcatttcatgtttgtttgtttctgt  
gatagggtcttgctttgtcgtccaggcttgagtgcatgtggtgtgatctcagctcactgcagcctc  
cacctcccaggctcattcgaacctcccgccttggccttccgagtcctgagactataggcatgcac  
caccacacccagtttaattttaaaattttttgtagagatgggggttttgctatgttaccagggtgg  
tcttgaactcctgggctcaagcagttctcctgccctggcttctcaaagccctgggattgcagggtg  
tgagccactgcacctggcacagagtcattttggagggtttagggtcccaggaattatcccaggggc  
tgcacatggcctggaactcttaacagaaaagggtgtctcccaattggaaaggctctaggcctttcag  
ttaagttgataaatttccctcctagagaagagaatagccacttctacaagcataaacaggtaacagga  
ggaggaagtggtggctccgggagcctggatctgaggccttggccttctaggccccaggagaaactaga  
acgttggtccatgcaagctatccagggtatccttggtatccttcagatgtgcttagcagaggccaac  
ttccacacacttggtctaaaattttctcccttctcctcttcatctgccttccccaggcagcct  
cctccttccccagggtcttcacatcagggtttggcctttatgctccatccagctcatctgtcactt  
gtcacctgaagcccacagtcctcgtcctctctgcactctagggcacttactaagtggatgtgg  
cctcctgagagtggtttttgttggtgttcccttttttatggccacttaagtgtttatatttgcttt  
atttgtatttacctctctgtatcataaattccatacagggtggctgggagcagtgactcacatctg  
taatcccagtcatttggaaggctgaggtgggaggatcgcttgaggccaagagttcgagactagcc  
tgggcaatatagcgagaccctctatctacaaaaaaacattccttacaggttaagtgaagg  
agttgtattacaacctccctatcatctactcagagccagtgctcatttgatcttgctaaatta  
gttactgagaataatgacaatatcctcttcatgagagagttttgacattaggcctgctgtccag  
aagtgcatttttaattctttccctcaacaaatcatttaacattttgaaaagtagtttatgtttt  
ttggaaaaaatgtaagacactaaaggaggacatgaaagtacctcctaaagttcctgctaaaagga  
ggaagtgaagtagctccctttgtgttttccaaaataacctttcctttctagccttttggtctat  
gtatgttcaaagatatgcaaacagaatagcattcaagcagtggtcttaaaaatattgtaatcac  
atactttacatgtctcctttagggtttctccatcttgatgctgttgacattttgggtccaagtgt  
tctttattatggtagggctgtcctgtgcactcatagacgggttagccgcactctctgcctgtacct  
cccagtggtgaggtcaaaaatgcctccggacatggccagggtgccccatggagagtgaaatcac  
tggatagtagtaatgtcaacacctagaagccctcaagtgctgactgcatgccatgtgtattct  
cactttttccctgtgttaactcactcagcctcacaaccactctatacgatctctactgttaacgt  
tcaccagtgagaaaactcagacccaaagaacttaagcctgttgcccagggtcacctgctggtgg  
gtgatacaaacctgcccaggctgagtcgggagtagatgtcaatgctgtgttcttctccctcctca  
ttctacctcatttctccctacaagctgcacaacatctcgaatagatatcacaatatatttcatcag  
ttgtttctgatctaaatttggtcagattttacattaggataataaccacaatgcatgctgcaatgt  
ataaagctttgtgtgtatatccttgacactgtagggttaaattttctagaagtctgattgtcttaa  
aatgaagcacattaaaaatttgggcaggcacatccaaactgccttcaaggaatttttttttta  
aatgttctttctgttctattcttctcctaattgattcttctgcctcactggcacaagtgggtccta  
ccctgtttacaccaaggagctttggtgctttatccagaccacttctgggttctaaggaccattgag  
agacttctgaactttcagtcacttaacttgggtccctcacaagttaactgagagcaaagtactg  
aacacatttttaattgtgcagtcagtgactgtttcaggcttcaaactaacttggtataacacactgt  
cagtggtgttcaagggaccctgggactagaggagaactgagaagcaggcattggccctttgtttt  
ccgtgggcccccatcttccatgaaatctgagggtcagcaaagggtggggaggagggtgggtcc  
tctacaggtagctgggctaagaaataggagcccagggtacaggatttgcattaaaaatgagtccta  
ttgaccttctgtggggtgacaggctgggcttgagcctggctgttttctgggttctcagcaagt  
gatcatctgcatagtggagagccttgggtgagctcccgtcctgtgaactctaaaacaatgtc  
tgccaagtaggctctcttgagtaaatcacttcttttttctttaggctgacccaaaatgaactc  
aacgatgaagtggcagagagtttggcagaaatgttgaaagtcacacagacgttaaaagtattg  
gtaactcagagagccttacaatttcagactgtgctacttttcaaaagtatttttgagataaaat  
ttacatactgtaaaattcactctcttaaagtatacaattcagagggttttttagtgcaaccatcacc  
acctaattctagaacattttcactcctcctccccactccaaaaagccctgggtatccattaaagcag  
tcactccctgtcctcctccccagaccctggcaaccactaatccgctttctgtctctatggatttg

FIG. 18 (7 f 10)

cctactctgggcatttcatataaatggaatcaagcaatatgtgaccttttgtctctgtgttctag  
catgtttcattcctttttatggctaaatgataattcactctaaggaaattttgcagtttattaat  
cagttgatgggacatttgggttgtttctactttttgactattatgcgtaatgctactgtgaacac  
tcctgttcatgcttttgggtgaacatatgttttcatctcttttgggaatatacctgggaatagaa  
tttctgggtcatatggcaattctgtaactttttgaggagccaccaaaactgttttctaaagtggat  
gtactattttacattctcgcagcaatgtatgtggattccaatttctccacatcctcaccaacac  
ttattattgtccatctttttaaattctagtataactagtggatgtgaagtaatatgtggttttga  
tttgcatttccctgatgacaacaatgttgaatgtctttttatgtgcctactgggagtcgtatag  
cttctttggagaaatgtctccatatcctttgcccatttttaaatttgggtttgtcttctaatgctg  
agttataggggttctctatatattctgggtgctagaccttactagatacagggttttgcaagtat  
tttctttctttctgtggagtttttctctttcttgatagtgccttttaaaggacaacagttttta  
atttttgttttttttgagatggagtcttgccttctgtcaccagacaggagtgagtggtgatgatc  
tcagctctctgcaacctccacctcctgggttcaagcgattcttctgcctcagcctcctgagtagt  
tgggattacaggcatcagccaccatgcctgtctcattttgtatttttaatatagagatggggtttca  
ccatttaggcccaggctggtcttgaactcctgacctcaggtgatccacctgcctcagcctcccaa  
agtgtctgggattacaggcgaaaagccactgcacctggccaatagtttttaattttgatgaagtcc  
aatttatctatttttttcttttgggttgccttgccttgccttgccttgccttgccttgccttgc  
atccaagatcacaaagaactccacctaaagttttctgttaagcgttatagttgtttccctcacat  
ataggtctgcaatccattttgagtttaattttgtatagtgtaaagtgaggggttaacctcattctc  
ttgcacgtggatatccagctgtcccggcagcaccagtggtgaacagattatcttttctattga  
atggccttgacaccttgtcaaaaatcaattgaccataaatgtatgggtttatcttgaattctc  
tgttctgggtccattgatttataatgtctctcctatgccaggaccattgctgtagctttgtgtagta  
cattttgaaatcaggaggtgtgagttctactttgttcttcttctcaagattgtttagaccattc  
tgggttctttgtcatttcttgaattcagactcacttgccttgccttgccttgccttgccttgc  
tgctacatatgttttttcttttcttttagcctgcagaattatttgatcccttccctaaagtgc  
aggccagcctctccaggagagcagagctaggacaggggtcagaaagagagtcctgggtgctttgt  
gcattcccaacctgcactggccctagtgaaggcagcccgagtggtggatgtgcctggacactgc  
aggcttttttaggggcattaggtgctctccttctgacctcctgccacatcttgggtggaggtgc  
cttccctgccttcaaaaaagcctaagtgtgactagaaaacagcagagtgtaactgaatacagaa  
cttgggtgcccacttccctggttctatttttgccttttgaaaggggaaggtcattacctctgccat  
tgaaccagggggccctagccctgtgggggtatggctgggagcaccagatcctggctgcagcccag  
ccaccagtgtgctgtgtgcttgggcagtaaacagtgacaagagctcccttccccctggacactgt  
gcctaataacctcctcttgaatctcacacaccagtcagtggtggggggcactcttatagttattct  
cagtttacagatgacacaactgaggcagacagatgcgtttatttcttcaaggttctgtagctg  
aacagtgggggagggaggggtttaaaggagagctgcacctgcctctgcaatactgcctctcacgagggga  
gtcctcttcattcatgacagcatagggccctcgtcttctcctggtaagggcttcttcttgggtcag  
tgccaggatttctaagggtcatgttttagcaggagcctattctacaaacagccaggagcaggggaat  
gactctgtgatgaagcggagacactacagcctcttgatgcatttatttcttgggttgggttagaag  
cgtagctgcccaggagcatttcaggagaggcctggcttcttagcgatagctgaaaactttgtt  
tcatttgaatcactgctacccagaacaatgggggtgcattctcagagtcctccattattaaagcttt  
tccactgagccccatgagaactattcatgagaactattcatggcagcataactgtttctcctcc  
ctccctcttgcatgttggtagcctcttaactttaaaacctgccttgcctttccctagctacctgg  
aaggagacgtcagacttccctgtcccattggtgtgtttcttacaatttgttgttcagattgggtggtc  
tcccaaatatataaaaaatataaatggagtctcactctgtcaccaggttgagtgagtgagtgga  
cgatcttgggtcactgcaacctccacctccagttcaagcaattctcctacctcagtcctccgag  
tagctgggagtagaggtgcacaccaccatgccagctaattttttgtatttttaatatagagacag  
gttggccaggatggtatcgatctcctgagctcgtgatccaccacctcggcctcccaaagtgtg  
ggattacaggtgtgagccactgcacctggcccaaatattttgattatgcacctctgcagtgaaa  
aatgcaaacacacacatcagttcatgtattacattatgttactataaaaaacaaacagaaaattt  
aaaaaatatacagctatccttactctagtggatcttacctggacacttttagccagatacaaaag  
tcacatggactcagttcttccctgaccaacttgtctcttcttcccaaaacaccttgcaactccc  
ttaagaaggggtcaaatttgatccagttatgtgattttatacaagttatgttcttcttccaggc  
ttatccagaatcagatcacagctaaggggactgccagctggcagatgcgttacagagcaacact  
ggcataacagagatttggttaagatcccagcgtttgtcacagtaataacaccagtgactgtttact

FIG. 18 (8 of 10)



caccaccactgactgtgcaaggcacaacgcaggggtggtttctgtttattcctccagcaaccctgc  
acagtaatggtattacctctgttttacagaggttagacagaggcccagaccagtgaataaaggttg  
cccaaggtcactacgagagaagctagaattcagcccagaatgcctgattccatattctgtgctct  
cctgccctgggcccccgccctcatctaccttcattgggtgggatgggggaagtggccagtgaat  
gatttcctagtgggaagtaaatccccctgggactcagcaattgagagatgactgtgtggccagga  
gtttggagctcattcttccccctttctgggttcgctaagacatttcagggctgacttgaactgac  
ctgtgctctttgtctacttctttttctgctttgagaacttccttatgctaataagaagaaaaaa  
gtttgctttactgtgacattgagcgccatgccacttcttcttgcctcccataaggcacagacac  
tccccactcagcagctcccttaacaacttaattgcctgggtgacgtgggactgggtggatgctgg  
gagagggggccttattaactatgtcctcctttcatgactggggagaatttcatagcccaattaaaaa  
aaaacaaaaaacagctccttggccaacacaggctcctcatacagtgttttttaactttgcttta  
gaacttggttggaacttgatcataaaatcgatcagtttggtgaattgcaaccaacaatatttaaaa  
agaaaacagaacagaacaaaatatcaggatgcaatgtgcatggtatgaaagtatcatttcattca  
tcttagttcatgtctgcatgtgagtggtgtgtgttgcataagtgttggttcacaacataaaat  
gtaattcttatttagggttgtagacaaaaggttttttttaaaaaaacactgttggttaggcat  
ggtggctcatgcctgtaataaccagcactttgggaggccaagatgggcagatctcttgagcacagg  
agtttgagaccagcctgggcaacatgcaaaacccgtcactacaaaaatagcccgacatgggtgc  
tatgtgctgtagtcccagctactcaggaagctgatgtgggaggatggatgcatgggagatcaag  
gctgcagtgagccaggatcatgccactgcaatccagcctgggtgccagagaccctgtctcaaaaa  
acaaaaaagaaaaaaagaaaaacacccatcatagagaatagagcccagatctaaacagacacctgt  
ggcctgtgtgctgcaagcccagcctgccagcagcctgggaagcactggagggcactggaact  
gtttgcatgggtgtttgcctcagggcactccgtttctgctgattcttaagtttgaggacagca  
ggcagagggggagaggaaggagactgccagactacagaacagtttgagagcacagttggcttcc  
acttttctctgtagctgggtcaggcgggttagtaaacactacagttgctttaatctgtcaagttt  
caaaatctgcattgcttccctcttgagggtcaccattcctacacaaggaaccatttttagtagggc  
caggagacttcagcttcaaggcctgcacttggtgcagggtggagaggggaactggccaccaattc  
agagagggcaggacaggcggcatgggtgctggtcttgggagtgtcttcacttaggtccctggctt  
gttctgggagcctccagagcatgctcctctgtgtgtgacttcatgggactgggctctgagaaggc  
tgtggctttgttggccctgccagggactgccacaccaggccacagggttggtggttgagctggccg  
gggagccacgttcagggagcagctctgcttggagccaacacttacagagtaagccttctccttgg  
acttgtaactgtactgacacttatttctacctcattcctttctgaaaaataacttggaaagtctga  
agtccttgatgagttctgtctttaagaacagaaattagaggtgaacaatgaacactgtaaat  
cagaaatgtatcccactccagtataacagctttctgtgaggctatctcctccagactgtggctct  
gggaggggtggggcctgagtcagggtccttagggactagtgctgtgtcttcttattcttgaata  
acgaaacgcttgagcatcagggactgtgctagcaccaaaaaatccagtggtgaacaacatggctc  
atgggttcactgtctagaaagggaagacacattaaagaaaaaatcatttgcgtaattatttaaat  
tacaactgtgatgggtactatcacaaagggggaaggccaagaggggaacctgatttagatgaggttg  
caggggaaggcctctctgaggaagcagcacttacactaagccatgaaggatgaataggagctagtc  
agctgaggtgagtattctgcgtagggaacagcatgtgcaaagggtctggggcaggagggagtg  
gtgtcctggaagaactgccagaagctgctgtgccccagggttcagacagtggtggaagaggggact  
acaggaggctgaggagataggcagggactggaccataaaaagatctgtgggtcatgatgtgcat  
tggtctttatcctaaaagtgatggaaagtcagtgaacagtttgaagcaggagaggcatgtgatca  
gatctgcaatgcaaaaagaccaattcttggtcttcttaggaaactgaattggagaaggccagagt  
acgtggaaatgacctgtcagtaggacattgtactgatgcagggaagagatgatgggtgctcagac  
caagatggccggccaaagacatagaggttcaggaggagcattctagattcttaggaattaggggga  
gaactttgtgatacaaggaacatggggatgagaaggaaggtgtccaggttgacccccagggttact  
aacctgctcagcaggatgagagtggtccattcactaagccagggggaccctaggaggtgtggctac  
tttgaggtgtgggggagaggtccaagtgaggatgccaaagcaggttaactgcctccacggacataca  
aacaaggccgtggcattgatgagatcggttggggaaaagggttagccccaacctggaggaaat  
ctcagatgtagaggtcacatggaggagaatataggaaaggaaattgaagtagagtgctcagatgc  
aggagaaaaatcagcgcataataaccaagccaagggggagggagtgctcaagaaggagggagagga  
gaggtcaggacagccaaaatcctgagggccaagaaagacaagacctggaaaatgtcattaaattc  
aggcttaggaggtacaggtgaccttagtgagaccagtgaaacagagggatggcagctggagag  
gatccatgctaataatgaaggaaactatctgcaaagggtatgttccttaatttcagggatacatgtg

tattgtgtgatacacgagtgtgtgctatgaacacaccttgggaaggagtgtgcgaggatccttaa  
cattttacctgtgtacttttgtcttcctccttttcaacagcctaaatggaaacctgataaaacca  
gaggaggccaaagtctatgaagatgagaagcggattatctgtttctgagaggatgctttcctgtt  
catgggggttttgccttgagcctcagcagcaaatgccactctgggcagtcttttgtgtcagtgt  
cttaaaggggcctgcgaggcgggactatcaggagtccactgcctccatgatgcaagccagcttc  
ctgtgcagaaggctctggtcggcaaactccctaagtaccgcgtacaattctgcagaaaaagaatgt  
gtcttgcgagctgtttagttacagtaaatacactgtgaagagactttattgcctattataa

FIG. 18 (10 of 10)

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/02544**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C07H 21/02, 21/04; C12N 15/11; C12Q 1/68; C07K 5/00

US CL : 435/6; 536/23.1; 530/300

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/23.1; 530/300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONEElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
APS, MEDLINE, BIOSIS, EMBASE, CAPLUS, SCISEARCH**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HOFMANN et al. The CARD Domain: A New Apoptotic Signalling Motif. TIBS. May 1997, Vol. 22, pages 155-156, see entire document.	15, 17-19, 21-22
A,P	McCARTHY et al. RIP2 is a Novel NF-kB-Activating and Cell Death-Inducing Kinase. The Journal Of Biological Chemistry. 03 July 1998, Vol. 273, No. 27, pages 16968-16975, see entire document.	15, 17-19, 21-22
A,E	YAN et al. mE10, a Novel Caspase Recruitment Domain-Containing Proapoptotic Molecule. The Journal Of Biological Chemistry. 09 April 1999, Vol. 274, No. 15, pages 10287-10292, see entire document.	15, 17-19, 21-22



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

19 MAY 1999

Date of mailing of the international search report

26 MAY 1999

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

SEAN McGARRY

Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/02544

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☒ Claims Nos.: 1-14, 16, and 20  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
  
These claims rely upon specific nucleotide and amino acid sequences that define the invention. No computer readable form of the sequence listing was filed in this application
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

CORRECTED VERSION

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
12 August 1999 (12.08.1999)

PCT

(10) International Publication Number  
**WO 99/40102 A1**

(51) International Patent Classification<sup>6</sup>: **C07H 21/02**,  
21/04, C12N 15/11, C12Q 1/68, C07K 5/00

(74) Agents: **MEIKLEJOHN, Anita, L.** et al.; Fish &  
Richardson P.C., 225 Franklin Street, Boston, MA  
02110-2804 (US).

(21) International Application Number: PCT/US99/02544

(22) International Filing Date: 5 February 1999 (05.02.1999)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
09/019,942 6 February 1998 (06.02.1998) US  
09/099,041 17 June 1998 (17.06.1998) US  
09/207,359 8 December 1998 (08.12.1998) US

(81) Designated States (*national*): AL, AM, AT, AU, AZ, BA,  
BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES,  
FI, GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG,  
KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK,  
MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI,  
SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW.

(63) Related by continuation (CON) or continuation-in-part  
(CIP) to earlier applications:

US 09/207,359 (CON)  
Filed on 8 December 1998 (08.12.1998)  
US 09/099,041 (CON)  
Filed on 17 June 1998 (17.06.1998)  
US 09/019,942 (CON)  
Filed on 6 February 1998 (06.02.1998)

(84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ,  
BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE,  
CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC,  
NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA,  
GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**  
— with international search report

(48) Date of publication of this corrected version:  
20 September 2001

(71) Applicant (*for all designated States except US*): **MIL-  
LENNIUM PHARMACEUTICALS, INC.** [US/US]; 75  
Sidney Street, Cambridge, MA 02139 (US).

(15) Information about Correction:  
see PCT Gazette No. 38/2001 of 20 September 2001, Sec-  
tion II

(72) Inventor; and

(75) Inventor/Applicant (*for US only*): **BERTIN, John**  
[US/US]; Apartment 1, 475 Arsenal Street, Watertown,  
MA 02172 (US).

*For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.*

(54) Title: NOVEL MOLECULES OF THE CARD-RELATED PROTEIN FAMILY AND USES THEREOF

(57) Abstract: Novel CARD-3, CARD-4L, CARD-4S, CARD-4Y, CARD-4Z, and murine CARD-4L polypeptides, proteins, and nucleic acid molecules are disclosed. In addition to isolated CARD-3, CARD-4L, CARD-4S, CARD-4Y, CARD-4Z, and murine CARD-4L proteins, and the invention further provides CARD-3, CARD-4L, CARD-4S, CARD-4Y, CARD-4Z, and murine CARD-4L fusion proteins, antigenic peptides and anti-CARD-3, anti-CARD-4L and anti-CARD-4S, anti-CARD-4Y, anti-CARD-4Z, and anti-murine CARD-4L antibodies. The invention also provides CARD-3, CARD-4L, CARD-4S, CARD-4Y, CARD-4Z, and murine CARD-4L nucleic acid molecules, recombinant expression vectors containing a nucleic acid molecule of the invention, host cells into which the expression vectors have been introduced and non-human transgenic animals in which a CARD-3, CARD-4L, CARD-4S, CARD-4Y, CARD-4Z, and murine CARD-4L gene has been introduced or disrupted. The invention further provides CARD-3 and CARD-4 target proteins that bind to CARD-3 or CARD-4 and allelic variants of human CARD-4. Diagnostic, screening and therapeutic methods utilizing compositions of the invention are also provided.

WO 99/40102 A1

NOVEL MOLECULES OF THE CARD-RELATED PROTEIN  
FAMILY AND USES THEREOF

5           Cross Reference to Related Applications

This application is a continuation-in-part of U.S. Application Serial No. 09/207,359 filed December 8, 1998, which is a continuation-in-part of U.S. Application Serial No. 09/099,041, filed June 17, 1998, which is a  
10 continuation-in-part of U.S. Application Serial No. 09/019,942, filed February 6, 1998. The contents of each of these applications is incorporated herein by this reference.

Background of the Invention

15           In multicellular organisms, homeostasis is maintained by balancing the rate of cell proliferation against the rate of cell death. Cell proliferation is influenced by numerous growth factors and the expression of proto-oncogenes, which typically encourage progression  
20 through the cell cycle. In contrast, numerous events, including the expression of tumor suppressor genes, can lead to an arrest of cellular proliferation.

In differentiated cells, a particular type of cell death called apoptosis occurs when an internal suicide  
25 program is activated. This program can be initiated by a variety of external signals as well as signals that are generated within the cell in response to, for example, genetic damage. For many years, the magnitude of apoptotic cell death was not appreciated because the  
30 dying cells are quickly eliminated by phagocytes, without an inflammatory response.

The mechanisms that mediate apoptosis have been intensively studied. These mechanisms involve the activation of endogenous proteases, loss of mitochondrial

- 2 -

function, and structural changes such as disruption of the cytoskeleton, cell shrinkage, membrane blebbing, and nuclear condensation due to degradation of DNA. The various signals that trigger apoptosis are thought to  
5 bring about these events by converging on a common cell death pathway that is regulated by the expression of genes that are highly conserved from worms, such as *C. elegans*, to humans. In fact, invertebrate model systems have been invaluable tools in identifying and  
10 characterizing the genes that control apoptosis. Through the study of invertebrates and more evolved animals, numerous genes that are associated with cell death have been identified, but the way in which their products interact to execute the apoptotic program is poorly  
15 understood.

Caspases, a class of proteins central to the apoptotic program, are cysteine protease having specificity for aspartate at the substrate cleavage site. These proteases are primarily responsible for the  
20 degradation of cellular proteins that lead to the morphological changes seen in cells undergoing apoptosis. For example, one of the caspases identified in humans was previously known as the interleukin-1 $\alpha$  (IL-1 $\alpha$ ) converting enzyme (ICE), a cysteine protease responsible for the  
25 processing of pro-IL-1 $\alpha$  to the active cytokine. Overexpression of ICE in Rat-1 fibroblasts induces apoptosis (Miura et al., Cell 75:653, 1993).

Many caspases and proteins that interact with caspases possess domains of about 60 amino acids called a  
30 caspase recruitment domain (CARD). Hofmann et al. (TIBS 22:155, 1997) and others have postulated that certain apoptotic proteins bind to each other via their CARDS and that different subtypes of CARDS may confer binding specificity, regulating the activity of various caspases,  
35 for example.

- 3 -

The functional significance of CARDS have been demonstrated in recent publications. Duan et al. (Nature 385:86, 1997) showed that deleting the CARD at the N-terminus of RAIDD, a newly identified protein involved in apoptosis, abolished the ability of RAIDD to bind to caspases. In addition, Li et al. (Cell 91:479, 1997) showed that the N-terminal 97 amino acids of apoptotic protease activating factor-1 (Apaf-1) was sufficient to confer caspase-9-binding ability. Inohara et al. (J. Biol. Chem. 273:12296-12300, 1998) showed that Apaf-1 can bind several other caspases such as caspase-4 and caspase-8. Apaf-1 can interact with caspases via CARD-CARD interaction (Li et al., supra, Hu et al., PNAS, 95:4386-4391, 1998).

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) is a transcription factor expressed in many cell types and which activates homologous or heterologous genes that have  $\kappa$ B sites in their promoters. Quiescent NF- $\kappa$ B resides in the cytoplasm as a heterodimer between proteins referred to as p50 and p65 and is complexed with the regulatory protein I $\kappa$ B. NF- $\kappa$ B binding to I $\kappa$ B causes NF- $\kappa$ B to remain in the cytoplasm. At least two dozen stimuli that activate NF- $\kappa$ B are known (New England Journal of Medicine 336:1066, 1997) and they include cytokines, protein kinase C activators, oxidants, viruses, and immune system stimuli. NF- $\kappa$ B activating stimuli activate specific I $\kappa$ B kinases that phosphorylate I $\kappa$ B leading to its degradation. Once liberated from I $\kappa$ B, NF $\kappa$ B translocates to the nucleus and activates genes with  $\kappa$ B sites in their promoters. How all of these NF- $\kappa$ B activating stimuli act is unknown at the present time and it is presumed that novel NF- $\kappa$ B pathway components are involved. NF- $\kappa$ B and the NF- $\kappa$ B pathway has been implicated in mediating chronic inflammation in inflammatory diseases such as asthma, ulcerative colitis, rheumatoid arthritis (New



- 4 -

England Journal of Medicine 336:1066, 1997) and inhibiting NF- $\kappa$ B or NF- $\kappa$ B pathways may be an effective way of treating these diseases. NF- $\kappa$ B and the NF- $\kappa$ B pathway has also been implicated in atherosclerosis  
5 (American Journal of Cardiology 76:18C, 1995), especially in mediating fatty streak formation, and inhibiting NF- $\kappa$ B or NF- $\kappa$ B pathways may be an effective therapy for atherosclerosis.

#### Summary of the Invention

10 The present invention is based, at least in part, on the discovery of genes encoding CARD-3 and CARD-4. The CARD-4 gene can express a long transcript that encodes CARD-4L, a short transcript that encodes partial CARD-4S, or two CARD-4 splice variants. A murine full  
15 length cDNA sequence for the murine ortholog of CARD-4L is also presented. CARD-3 and CARD-4 are intracellular proteins that are predicted to be involved in regulating caspase activation. CARD-4 is found to activate the NF- $\kappa$ B pathway and to enhance caspase 9-mediated cell  
20 death. In addition, proteins that bind to CARD-4 are presented including CARD-3 and hNUDC.

The CARD-3 cDNA described below (SEQ ID NO:1) has a 1620 open reading frame (nucleotides 214 to 1833 of SEQ ID NO:1; SEQ ID NO:3) which encodes a 540 amino acid  
25 protein (SEQ ID NO:2). CARD-3 contains a kinase domain which extends from amino acid 1 to amino acid 300 of SEQ ID NO:2; SEQ ID NO:4, followed by a linker domain at amino acid 301 to amino acid 431 of SEQ ID NO:2; SEQ ID NO:5 and a CARD at amino acid 432 to amino acid 540 of  
30 SEQ ID NO:2; SEQ ID NO:6.

At least four forms of CARD-4 exist in the cell, a long form, CARD-4L, a short form, CARD-4S, and two splice variants, CARD-4Y and CARD-4Z. The cDNA of CARD-4L described below (SEQ ID NO:7) has a 2859 nucleotide open

- 5 -

reading frame (nucleotides 245-3103 of SEQ ID NO:7; SEQ ID NO:9) which encodes a 953 amino acid protein (SEQ ID NO:8). CARD-4L protein possesses a CARD domain (amino acids 15-114; SEQ ID NO:10). The nucleotide sequence of the full length cDNA corresponding to the murine ortholog of human CARD-4L is presented (SEQ ID NO:42) as is the predicted amino acid sequence of murine CARD-4L (SEQ ID NO:43). A comparison between the predicted amino acid sequences of human CARD-4L and murine CARD-4L is also depicted in Figure 17.

Human CARD-4L is also predicted to have a nucleotide binding domain which extends from about amino acid 198 to about amino acid 397 of SEQ ID NO:8; SEQ ID NO:11, a Walker Box "A", which extends from about amino acid 202 to about amino acid 209 of SEQ ID NO:8; SEQ ID NO:12, a Walker Box "B", which extends from about amino acid 280 to about amino acid 284, of SEQ ID NO:8; SEQ ID NO:13, a kinase 1a (P-loop) subdomain, which extends from about amino acid 127 to about amino acid 212 of SEQ ID NO:8; SEQ ID NO:46, a kinase 2 subdomain, which extends from about amino acid 273 to about amino acid 288 of SEQ ID NO:8; SEQ ID NO:47, a kinase 3a subdomain, which extends from about amino acid 327 to about amino acid 338 of SEQ ID NO:8; SEQ ID NO:14, and ten Leucine-rich repeats which extend from about amino acid 674 to about amino acid 950 of SEQ ID NO:8. The first Leucine-rich repeat extends from about amino acid 674 to about amino acid 701 of SEQ ID NO:8; SEQ ID NO:15. The second Leucine-rich repeat extends from about amino acid 702 to about amino acid 727 of SEQ ID NO:8; SEQ ID NO:16. The third Leucine-rich repeat extends from about amino acid 728 to about amino acid 754 of SEQ ID NO:8; SEQ ID NO:17. The fourth Leucine-rich repeat extends from about amino acid 755 to about amino acid 782 of SEQ ID NO:8; SEQ ID NO:18. The fifth Leucine-rich repeat extends from about

- 6 -

amino acid 783 to about amino acid 810 of SEQ ID NO:8;  
SEQ ID NO:19. The sixth Leucine-rich repeat extends from  
about amino acid 811 to about amino acid 838 of SEQ ID  
NO:8; SEQ ID NO:20. The seventh Leucine-rich repeat  
5 extends from about amino acid 839 to about amino acid 866  
of SEQ ID NO:8; SEQ ID NO:21. The eighth Leucine-rich  
repeat extends from about amino acid 867 to about amino  
acid 894 of SEQ ID NO:8; SEQ ID NO:22. The ninth  
10 Leucine-rich repeat extends from about amino acid 895 to  
about amino acid 922 of SEQ ID NO:8; SEQ ID NO:23 and the  
tenth leucine-rich repeat extends from about amino acid  
923 to about amino acid 950 of SEQ ID NO:8; SEQ ID NO:24.

The partial cDNA of CARD-4S described below (SEQ  
ID NO:25) has a 1470 nucleotide open reading frame  
15 (nucleotides 1-1470 of SEQ ID NO:25; SEQ ID NO:27) which  
encodes a 490 amino acid protein (SEQ ID NO:26). CARD-4S  
protein possesses a CARD domain (amino acids 1-74 of SEQ  
ID NO:26; SEQ ID NO:28). CARD-4S is predicted to have a  
P-Loop which extends from about amino acid 163 to about  
20 amino acid 170 of SEQ ID NO:26; SEQ ID NO:29, and a  
Walker Box "B" which extends from about amino acid 241 to  
about amino acid 245 of SEQ ID NO:26; SEQ ID NO:30.

A human CARD-4Y nucleotide cDNA sequence is  
presented (SEQ ID NO:38) as is the amino acid sequence of  
25 the predicted CARD-4Y product (SEQ ID NO:39). A human  
CARD-4Z nucleotide cDNA sequence is presented (SEQ ID  
NO:40) as is the amino acid sequence of the predicted  
CARD-4Z product (SEQ ID NO:41). A comparison of the  
CARD-4Y, CARD-4Z, and human CARD-4L predicted amino acid  
30 sequences is also shown in Figure 14.

Like other proteins containing a CARD domain, both  
CARD-3 and CARD-4 are expected to participate in the  
network of interactions that lead to caspase activity.  
Human CARD-4L is expected to play a functional role in  
35 caspase activation similar to that of Apaf-1 (Zou et al.,

- 7 -

Cell, 90:405-413, 1997). For example, upon activation, CARD-4L might bind a nucleotide, thus allowing CARD-4L to bind and activate a CARD-containing caspase via a CARD-CARD interaction, leading to apoptotic death of the  
5 cell. Accordingly, CARD-3 and CARD-4 molecules are useful as modulating agents in regulating a variety of cellular processes including cell growth and cell death. In one aspect, this invention provides isolated nucleic acid molecules encoding CARD-3 or CARD-4 proteins or  
10 biologically active portions thereof, as well as nucleic acid fragments suitable as primers or hybridization probes for the detection of CARD-3 or CARD-4 encoding nucleic acids.

The invention encompasses methods of diagnosing  
15 and treating patients who are suffering from a disorder associated with an abnormal level or rate (undesirably high or undesirably low) of apoptotic cell death, abnormal activity of the Fas/APO-1 receptor complex, abnormal activity of the TNF receptor complex, or  
20 abnormal activity of a caspase by administering a compound that modulates the expression of CARD-3 or CARD-4 (at the DNA, mRNA or protein level, e.g., by altering mRNA splicing) or by altering the activity of CARD-3 or CARD-4. Examples of such compounds include  
25 small molecules, antisense nucleic acid molecules, ribozymes, and polypeptides.

Certain disorders are associated with an increased number of surviving cells, which are produced and continue to survive or proliferate when apoptosis is  
30 inhibited. These disorders include cancer (particularly follicular lymphomas, carcinomas associated with mutations in p53, and hormone-dependent tumors such as breast cancer, prostate cancer, and ovarian cancer), autoimmune disorders (such as systemic lupus  
35 erythematosus, immune-mediated glomerulonephritis), and

- 8 -

viral infections (such as those caused by herpesviruses, poxviruses, and adenoviruses).

Failure to remove autoimmune cells that arise during development or that develop as a result of somatic  
5 mutation during an immune response can result in autoimmune disease. One of the molecules that plays a critical role in regulating cell death in lymphocytes is the cell surface receptor for Fas.

Populations of cells are often depleted in the  
10 event of viral infection, with perhaps the most dramatic example being the cell depletion caused by the human immunodeficiency virus (HIV). Surprisingly, most T cells that die during HIV infections do not appear to be infected with HIV. Although a number of explanations  
15 have been proposed, recent evidence suggests that stimulation of the CD4 receptor results in the enhanced susceptibility of uninfected T cells to undergo apoptosis.

A wide variety of neurological diseases are  
20 characterized by the gradual loss of specific sets of neurons. Such disorders include Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS) retinitis pigmentosa, spinal muscular atrophy, and various forms of cerebellar degeneration. The cell loss  
25 in these diseases does not induce an inflammatory response, and apoptosis appears to be the mechanism of cell death.

In addition, a number of hematologic diseases are associated with a decreased production of blood cells.  
30 These disorders include anemia associated with chronic disease, aplastic anemia, chronic neutropenia, and the myelodysplastic syndromes. Disorders of blood cell production, such as myelodysplastic syndrome and some forms of aplastic anemia, are associated with increased  
35 apoptotic cell death within the bone marrow. These

- 9 -

disorders could result from the activation of genes that promote apoptosis, acquired deficiencies in stromal cells or hematopoietic survival factors, or the direct effects of toxins and mediators of immune responses.

5           Two common disorders associated with cell death are myocardial infarctions and stroke. In both disorders, cells within the central area of ischemia, which is produced in the event of acute loss of blood flow, appear to die rapidly as a result of necrosis.  
10 However, outside the central ischemic zone, cells die over a more protracted time period and morphologically appear to die by apoptosis.

          The invention features a nucleic acid molecule which is at least 45% (or 55%, 65%, 75%, 85%, 95%, or  
15 98%) identical to the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID:25, SEQ ID NO:27, SEQ ID NO:38, SEQ ID NO:40, and SEQ ID NO:42, the nucleotide sequence of the cDNA insert of the plasmid deposited with ATCC as Accession Number (the  
20 "cDNA of ATCC \_\_\_\_\_"), the nucleotide sequence of the cDNA insert of the plasmid deposited with ATCC as Accession Number (the "cDNA of ATCC\_\_\_\_\_"), the nucleotide sequence of the cDNA insert of the plasmid deposited with ATCC as Accession Number (the "cDNA of  
25 ATCC\_\_\_\_\_"), or a complement thereof.

          The invention features a nucleic acid molecule which includes a fragment of at least 150 (300, 325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1300, 1600 or 1931) nucleotides of the nucleotide  
30 sequence shown in SEQ ID NO:1, or SEQ ID NO:3, or the nucleotide sequence of the cDNA ATCC \_\_\_\_\_, or a complement thereof.

          The invention also features a nucleic acid molecule which includes a fragment of at least 150 (350,  
35 400, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1300,

- 10 -

1600, 1900, 2100, 2400, 2700, 3000, or 3382) nucleotides of the nucleotide sequence shown in SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO: 43 or the nucleotide sequence of the cDNA ATCC \_\_\_\_\_, or a complement thereof.

5 Also within the invention is a nucleic acid molecule which includes a fragment of at least 150 (350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1300, 1600, 1900, 2100, 2400, 2700, and 3080) nucleotides of the nucleotide sequence shown in SEQ ID NO:25, SEQ ID  
10 NO:27, SEQ ID NO:38, SEQ ID NO:40, or the nucleotide sequence of the cDNA ATCC \_\_\_\_\_, or a complement thereof.

The invention features a nucleic acid molecule which includes a nucleotide sequence encoding a protein  
15 having an amino acid sequence that is at least 45% (or 55%, 65%, 75%, 85%, 95%, or 98%) identical to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:26, SEQ ID NO:39, SEQ ID NO:41, and SEQ ID NO:43, or the amino acid sequence encoded by the cDNA of ATCC \_\_\_\_\_, the  
20 amino acid sequence encoded by the cDNA of ATCC \_\_\_\_\_, or the amino acid sequence encoded by the cDNA of ATCC \_\_\_\_\_.

In an embodiment, a CARD-3 nucleic acid molecule has the nucleotide sequence shown in SEQ ID NO:1, or SEQ ID NO:3, or the nucleotide sequence of the cDNA of ATCC  
25 \_\_\_\_\_. In another embodiment, a CARD-4L nucleic acid molecule has the nucleotide sequence shown in SEQ ID NO:7, or SEQ ID NO:9, or the nucleotide sequence of the cDNA of ATCC \_\_\_\_\_. In yet another embodiment, a CARD-4S nucleic acid molecule has the nucleotide sequence shown  
30 in SEQ ID NO:25, or SEQ ID NO:27, or the nucleotide sequence of the cDNA of ATCC \_\_\_\_\_. In another embodiment, a murine CARD-4L nucleic acid molecule has the nucleotide sequence shown in SEQ ID NO:42. In another embodiment, a CARD-4Y nucleic acid molecule has

- 11 -

the nucleotide sequence shown in SEQ ID NO:38 or the nucleotide sequence of the cDNA of ATCC \_\_\_\_\_. In another embodiment, a CARD-4Z nucleic acid molecule has the nucleotide sequence shown in SEQ ID NO:40 or the  
5 nucleotide sequence of the cDNA of ATCC \_\_\_\_\_.

Also within the invention is a nucleic acid molecule which encodes a fragment of a polypeptide having the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:8 or SEQ ID NO:26 or SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID  
10 NO:43, the fragment including at least 15 (25, 30, 50, 100, 150, 300, 400 or 540, 600, 700, 800, 953) contiguous amino acids of SEQ ID NO:2 or SEQ ID NO:8 or SEQ ID NO:26 or SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID NO:43 or the polypeptide encoded by the cDNA of ATCC Accession Number \_  
15 \_\_\_\_\_, or the polypeptide encoded by the cDNA of ATCC Accession Number \_\_\_\_\_ or the polypeptide encoded by the cDNA of ATCC Accession Number \_\_\_\_\_.

The invention includes a nucleic acid molecule  
20 which encodes a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID NO:43 or an amino acid sequence encoded by the cDNA of ATCC Accession Number \_\_\_\_\_, wherein the nucleic acid molecule  
25 hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:38 or SEQ ID NO:40 or SEQ ID NO:42 under stringent conditions. The invention also includes a nucleic acid molecule which encodes a naturally occurring allelic variant of a polypeptide  
30 comprising the amino acid sequence of SEQ ID NO:8 or an amino acid sequence encoded by the cDNA of ATCC Accession Number \_\_\_\_\_, wherein the nucleic acid molecule hybridizes to a nucleic acid molecule comprising SEQ ID NO:7 or SEQ ID NO:9 under stringent conditions.



- 12 -

The invention also includes a nucleic acid molecule which encodes a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:26 or an amino acid sequence encoded by the cDNA of ATCC Accession Number \_\_\_\_\_, wherein the nucleic acid molecule hybridizes to a nucleic acid molecule comprising SEQ ID NO:25 or SEQ ID NO:27 under stringent conditions. In general, an allelic variant of a gene will be readily identifiable as mapping to the same chromosomal location as said gene. For example, in Example 6, the chromosomal location of the human CARD-4 gene is discovered to be chromosome 7 close to the SHGC-31928 genetic marker. Allelic variants of human CARD-4 will be readily identifiable as mapping to the human CARD-4 locus on chromosome 7 near genetic marker SHGC-31928.

Also within the invention are: an isolated CARD-3 protein having an amino acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98% identical to the amino acid sequence of SEQ ID NO:2; an isolated CARD-3 protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the kinase domain of SEQ ID NO:2 (e.g., about amino acid residues 1 to 300 of SEQ ID NO:2; SEQ ID NO:4); and an isolated CARD-3 protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the linker domain of SEQ ID NO:2 (e.g., about amino acid residues 301 to 431 of SEQ ID NO:2; SEQ ID NO:5); an isolated CARD-3 protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the CARD domain of SEQ ID NO:2 (e.g., about amino acid residues 432 to 540 of SEQ ID NO:2; SEQ ID NO:6); an isolated CARD-4L protein having an amino acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98% identical to the amino acid sequence of SEQ ID NO:8; an isolated

- 13 -

CARD-4L protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the CARD domain of SEQ ID NO:8 (e.g., about amino acid residues 15 to 114 of SEQ ID NO:8; SEQ ID NO:10); an isolated CARD-4L

5 protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the nucleotide binding domain of SEQ ID NO:8 (e.g., about amino acid residues 198 to 397 of SEQ ID NO:8; SEQ ID NO:11; an isolated CARD-4L protein having an amino acid sequence

10 that is at least about 85%, 95%, or 98% identical to the kinase 1a (P-loop) subdomain SEQ ID NO:8 (e.g., about amino acid 127 to about amino acid 212 of SEQ ID NO:8; SEQ ID NO:46); an isolated CARD-4L protein having an amino acid sequence that is at least about 85%, 95%, or

15 98% identical to the kinase 2 subdomain of SEQ ID NO:8 (e.g., about amino acid 273 to about amino acid 288 of SEQ ID NO:8; SEQ ID NO:47); an isolated CARD-4L protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to a kinase 3a subdomain of SEQ ID

20 NO:8 (e.g., about amino acid residues 327 to 338 of SEQ ID NO:8; SEQ ID NO:14); an isolated CARD-4L protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the Leucine-rich repeats of SEQ ID NO:8 (e.g., about amino acid residues 674 to 701 of

25 SEQ ID NO:8; SEQ ID NO:15; from amino acid 702 to amino acid 727 of SEQ ID NO:8; SEQ ID NO:16; which extends from amino acid 728 to amino acid 754 SEQ ID NO:8; SEQ ID NO:17; from amino acid 755 to amino acid 782 of SEQ ID NO:8; SEQ ID NO:18; from amino acid 783 to amino acid 810

30 of SEQ ID NO:8; SEQ ID NO:19; from amino acid 811 to amino acid 838 of SEQ ID NO:8; SEQ ID NO:20 from amino acid 839 to amino acid 866 of SEQ ID NO:8; SEQ ID NO:21; from amino acid 867 to amino acid 894 of SEQ ID NO:8; SEQ ID NO:22; from amino acid 895 to amino acid 922 of SEQ ID

35 NO:8; SEQ ID NO:23; and from amino acid 923 to amino acid

- 14 -

950 of SEQ ID NO:8; SEQ ID NO:24); an isolated CARD-4S protein having an amino acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98% identical to the amino acid sequence of SEQ ID NO:26; an isolated

5 CARD-4S protein having an amino acid sequence that is at least about 85%, 95%, or 98% identical to the CARD domain of SEQ ID NO:26 (e.g., about amino acid residues 1 to 74 of SEQ ID NO:26; SEQ ID NO:28). Also within the invention are: an isolated murine CARD-4L protein having an amino

10 acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98% identical to the amino acid sequence of SEQ ID NO:43. Also within the invention are: an isolated CARD-4Y protein having an amino acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98%

15 identical to the amino acid sequence of SEQ ID NO:39. Also within the invention are: an isolated CARD-4Z protein having an amino acid sequence that is at least about 65%, preferably 75%, 85%, 95%, or 98% identical to the amino acid sequence of SEQ ID NO:41.

20 Also within the invention are: an isolated CARD-3 protein which is encoded by a nucleic acid molecule having a nucleotide sequence that is at least about 65%, preferably 75%, 85%, or 95% identical to SEQ ID NO:3 or the cDNA of ATCC \_\_\_\_\_; an isolated CARD-3 protein which

25 is encoded by a nucleic acid molecule having a nucleotide sequence at least about 65% preferably 75%, 85%, or 95% identical to the kinase domain encoding portion of SEQ ID NO:1 (e.g., about nucleotides 213 to 1113 of SEQ ID NO:1); an isolated CARD-3 protein which is encoded by a

30 nucleic acid molecule having a nucleotide sequence at least about 65% preferably 75%, 85%, or 95% identical the linker domain encoding portion of SEQ ID NO:1 (e.g., about nucleotides 1114 to 1506 of SEQ ID NO:1); and an isolated CARD-3 protein which is encoded by a nucleic

35 acid molecule having a nucleotide sequence at least about

- 15 -

65% preferably 75%, 85%, or 95% identical the CARD domain encoding portion of SEQ ID NO:1 (e.g., about nucleotides 1507 to 1833 of SEQ ID NO:1); and an isolated CARD-3 protein which is encoded by a nucleic acid molecule  
5 having a nucleotide sequence which hybridizes under stringent hybridization conditions to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:3 or the non-coding strand of the cDNA of ATCC \_\_\_\_\_. Also within the invention are: an isolated CARD-4Y protein  
10 which is encoded by a nucleic acid molecule having a nucleotide sequence that is at least about 65%, preferably 75%, 85%, or 95% identical to SEQ ID NO:38 or the cDNA of ATCC \_\_\_\_\_. Also within the invention are nucleic acid molecules which include about nucleotides  
15 2759 to 2842 of SEQ ID NO:7; about nucleotides 2843 to 2926 of SEQ ID NO:7; about nucleotides 2927 to 3010 of SEQ ID NO:7; about nucleotides 3011 to 3094 of SEQ ID NO:7; and an isolated CARD-4L protein which is encoded by a nucleic acid molecule having a nucleotide sequence  
20 which hybridizes under stringent hybridization conditions to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:9 or the non-coding strand of the cDNA of ATCC \_\_\_\_\_.

Also within the invention are: an isolated CARD-4S  
25 protein which is encoded by a nucleic acid molecule having a nucleotide sequence that is at least about 65%, preferably 75%, 85%, or 95% identical to SEQ ID NO:27 or the cDNA of ATCC \_\_\_\_\_; an isolated CARD-3 protein which is encoded by a nucleic acid molecule having a nucleotide  
30 sequence at least about 65% preferably 75%, 85%, or 95% identical the CARD domain encoding portion of SEQ ID NO:25 (e.g., about nucleotides 1 to 222 of SEQ ID NO:25); an isolated CARD-3 protein which is encoded by a nucleic acid molecule having a nucleotide sequence at least about  
35 65% preferably 75%, 85%, or 95% identical the P-Loop

- 16 -

encoding portion of SEQ ID NO:25 (e.g., about nucleotides 485 to 510 of SEQ ID NO:25).

Also within the invention is a polypeptide which is a naturally occurring allelic variant of a polypeptide that includes the amino acid sequence of SEQ ID NO:2 or an amino acid sequence encoded by the cDNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, wherein the polypeptide is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:1 or SEQ ID NO:3 under stringent conditions.

Also within the invention is a polypeptide which is a naturally occurring allelic variant of a polypeptide that includes the amino acid sequence of SEQ ID NO:8 or an amino acid sequence encoded by the cDNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, wherein the polypeptide is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:7 or SEQ ID NO:9 under stringent conditions.

Also within the invention is a polypeptide which is a naturally occurring allelic variant of a polypeptide that includes the amino acid sequence of SEQ ID NO:26 or an amino acid sequence encoded by the cDNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, wherein the polypeptide is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule comprising SEQ ID NO:25 or SEQ ID NO:27 under stringent conditions.

Another embodiment of the invention features CARD-3 or CARD-4 nucleic acid molecules which specifically detect CARD-3 or CARD-4 nucleic acid molecules, relative to nucleic acid molecules encoding other members of the CARD superfamily. For example, in one embodiment, a CARD-4L nucleic acid molecule

- 17 -

hybridizes under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:7, SEQ ID NO:9, or the cDNA of ATCC \_\_\_\_\_, or a complement thereof. In another embodiment, the CARD-4L  
5 nucleic acid molecule is at least 300 (350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1300, 1600, 1900, 2100, 2400, 2700, 3000, or 3382) nucleotides in length and hybridizes under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence  
10 shown in SEQ ID NO:7, SEQ ID NO:9, the cDNA of ATCC \_\_\_\_\_, or a complement thereof. In another embodiment, an isolated CARD-4L nucleic acid molecule comprises nucleotides 287 to 586 of SEQ ID NO:7, encoding the CARD domain of CARD-4L, or a complement thereof. In yet  
15 another embodiment, the invention provides an isolated nucleic acid molecule which is antisense to the coding strand of a CARD-4L nucleic acid.

Another aspect of the invention provides a vector, e.g., a recombinant expression vector, comprising a  
20 CARD-3 or a CARD-4L nucleic acid molecule of the invention. In another embodiment the invention provides a host cell containing such a vector. The invention also provides a method for producing CARD-3 or CARD-4 protein by culturing, in a suitable medium, a host cell of the  
25 invention containing a recombinant expression vector such that a CARD-3 or CARD-4 protein is produced.

Another aspect of this invention features isolated or recombinant CARD-3 or CARD-4 proteins and polypeptides. Preferred CARD-3 or CARD-4 proteins and  
30 polypeptides possess at least one biological activity possessed by naturally occurring human CARD-3 or CARD-4, e.g., (1) the ability to form protein:protein interactions with proteins in the apoptotic signalling pathway; (2) the ability to form  
35 CARD-CARD interactions with proteins in the apoptotic

- 18 -

signaling pathway; (3) the ability to bind the CARD-3 or CARD-4 ligand; (4) and the ability to bind to an intracellular target. Other activities include: (1) modulation of cellular proliferation, (2) modulation of cellular differentiation and (3) modulation of cellular death (4) modulation of the NF- $\kappa$ B pathway.

The CARD-3 or CARD-4 proteins of the present invention, or biologically active portions thereof, can be operatively linked to a non-CARD-3 or non-CARD-4 polypeptide (e.g., heterologous amino acid sequences) to form CARD-3 or CARD-4 fusion proteins, respectively. The invention further features antibodies that specifically bind CARD-3 or CARD-4 proteins, such as monoclonal or polyclonal antibodies. In addition, the CARD-3 or CARD-4 proteins or biologically active portions thereof can be incorporated into pharmaceutical compositions, which optionally include pharmaceutically acceptable carriers.

In another aspect, the present invention provides a method for detecting the presence of CARD-3 or CARD-4 activity or expression in a biological sample by contacting the biological sample with an agent capable of detecting an indicator of CARD-3 or CARD-4 activity such that the presence of CARD-3 or CARD-4 activity is detected in the biological sample.

In another aspect, the invention provides a method for modulating CARD-3 or CARD-4 activity comprising contacting a cell with an agent that modulates (inhibits or stimulates) CARD-3 or CARD-4 activity or expression such that CARD-3 or CARD-4 activity or expression in the cell is modulated. In one embodiment, the agent is an antibody that specifically binds to CARD-3 or CARD-4 protein. In another embodiment, the agent modulates expression of CARD-3 or CARD-4 by modulating transcription of a CARD-3 or CARD-4 gene, splicing of a

- 19 -

CARD-3 or CARD-4 mRNA, or translation of a CARD-3 or CARD-4 mRNA. In yet another embodiment, the agent is a nucleic acid molecule having a nucleotide sequence that is antisense to the coding strand of the CARD-3 or CARD-4 mRNA or the CARD-3 or CARD-4 gene.

In one embodiment, the methods of the present invention are used to treat a subject having a disorder characterized by aberrant CARD-3 or CARD-4 protein or nucleic acid expression or activity by administering an agent which is a CARD-3 or CARD-4 modulator to the subject. In one embodiment, the CARD-3 or CARD-4 modulator is a CARD-3 or CARD-4 protein. In another embodiment the CARD-3 or CARD-4 modulator is a CARD-3 or CARD-4 nucleic acid molecule. In other embodiments, the CARD-3 or CARD-4 modulator is a peptide, peptidomimetic, or other small molecule.

The present invention also provides a diagnostic assay for identifying the presence or absence of a genetic lesion or mutation characterized by at least one of: (i) aberrant modification or mutation of a gene encoding a CARD-3 or CARD-4 protein; (ii) mis-regulation of a gene encoding a CARD-3 or CARD-4 protein; (iii) aberrant RNA splicing; and (iv) aberrant post-translational modification of a CARD-3 or CARD-4 protein, wherein a wild-type form of the gene encodes a protein with a CARD-3 or CARD-4 activity.

In another aspect, the invention provides a method for identifying a compound that binds to or modulates the activity of a CARD-3 or CARD-4 protein. In general, such methods entail measuring a biological activity of a CARD-3 or CARD-4 protein in the presence and absence of a test compound and identifying those compounds which alter the activity of the CARD-3 or CARD-4 protein.



- 20 -

The invention also features methods for identifying a compound which modulates the expression of CARD-3 or CARD-4 by measuring the expression of CARD-3 or CARD-4 in the presence and absence of a compound.

5 Other features and advantages of the invention will be apparent from the following detailed description and claims.

#### Brief Description of the Drawings

Figure 1 depicts the cDNA sequence (SEQ ID NO:1) of human CARD-3. The open reading frame of CARD-3 (SEQ ID NO:1) extends from nucleotide 213 to nucleotide 1833 nucleotide (SEQ ID NO:3).

Figure 2 depicts the predicted amino acid sequence (SEQ ID NO:2) of human CARD-3.

15 Figure 3 depicts the cDNA sequence (SEQ ID NO:7) of CARD-4L. The open reading frame of SEQ ID NO:7 extends from nucleotide 245 to nucleotide 3103 (SEQ ID NO:9).

Figure 4 depicts the predicted amino acid sequence (SEQ ID NO:8) of human CARD-4L.

Figure 5 depicts the partial cDNA sequence (SEQ ID NO:25) of CARD-4S and the predicted amino acid sequence (SEQ ID NO:25) of human CARD-4S. The open reading frame of CARD-4 (SEQ ID NO:25) extends from nucleotide 1 to 25 nucleotide 1470 (SEQ ID NO:27).

Figure 6 depicts the predicted amino acid sequence (SEQ ID NO:26) of human CARD-4S.

Figure 7 depicts an alignment of the CARD domains of CARD-4 (SEQ ID NO:10), CARD-3 (SEQ ID NO:6), ARC-CARD (SEQ ID NO:31), cIAP1-CARD (SEQ ID NO:32) and cIAP2-CARD (SEQ ID NO:33).

- 21 -

Figure 8 is a plot showing predicted structural features of human CARD-4L.

Figure 9 is a plot showing predicted structural features of human CARD-4S.

5        Figure 10 depicts the cDNA sequence (SEQ ID NO:38) of the human CARD-4Y splice variant clone. The predicted open reading frame of the human CARD-4Y splice variant clone extends from nucleotide 438 to nucleotide 1184.

10       Figure 11 depicts the amino acid sequence (SEQ ID NO:39) of the protein predicted to be encoded by the human CARD-4Y cDNA open reading frame.

15       Figure 12 depicts the cDNA sequence (SEQ ID NO:40) of the human CARD-4Z splice variant clone. The predicted open reading frame of the human CARD-4Z splice variant clone extends from nucleotide 489 to nucleotide 980.

Figure 13 depicts the amino acid sequence (SEQ ID NO:41) of the protein predicted to be encoded by the human CARD-4Z cDNA open reading frame.

20       Figure 14 depicts an alignment of human CARD-4L (SEQ ID NO:8), the predicted amino acid sequence of human CARD-4Y (SEQ ID NO:39), and the predicted amino acid sequence of human CARD-4Z (SEQ ID NO:41).

Figure 15 depicts the nucleotide sequence of the murine CARD-4L cDNA (SEQ ID NO:42).

25       Figure 16 depicts the predicted amino acid sequence of murine CARD-4L (SEQ ID NO:43).

Figure 17 depicts an alignment of human CARD-4L (SEQ ID NO:8) and the predicted amino acid sequence of murine CARD-4L (SEQ ID NO:43).

30       Figure 18 depicts a 32042 nucleotide genomic sequence of CARD-4.

#### Detailed Description of the Invention

The present invention is based, in part, on the discovery of cDNA molecules encoding human CARD-3, human

- 22 -

CARD-4 and partial murine CARD-4L proteins. A nucleotide sequence encoding a human CARD-3 protein is shown in Figure 1 (SEQ ID NO:1; SEQ ID NO:3 includes the open reading frame only). A predicted amino acid sequence of  
5 CARD-3 protein is also shown in Figure 2 (SEQ ID NO:2). CARD-4 has at least two forms, a long form, CARD-4L, and a short form, CARD-4S, as well as two or more splice variants. A nucleotide sequence encoding a human CARD-4L protein is shown in Figure 3 (SEQ ID NO:7; SEQ ID NO:9  
10 includes the open reading frame only). A predicted amino acid sequence of CARD-4L protein is also shown in Figure 4 (SEQ ID NO:8). A nucleotide sequence encoding a human CARD-4S protein is shown in Figure 5 (SEQ ID NO:25; SEQ ID NO:27 includes the open reading frame only). A  
15 predicted amino acid sequence of CARD-4S protein is also shown in Figure 6 (SEQ ID NO:26). Two additional splice variants of human CARD-4 are provided in Figures 10 and 11 (human CARD-4Y) and Figures 12 and 13 (human CARD-4Z) (predicted amino acid sequences: SEQ ID NO:39 and SEQ ID  
20 NO:41 and nucleic acid sequences: SEQ ID NO:38 and SEQ ID NO:40). These two splice variants are predicted to contain 249 and 164 amino acids, respectively. An alignment of human CARD-4Y, human CARD-4Z and human CARD-4L is shown in Figure 14.

25 In addition to the human CARD-4 proteins, a full length nucleotide sequence of the murine ortholog of human CARD-4L is provided in Figure 15 (SEQ ID NO:42). An alignment of murine CARD-4L with human CARD-4L is shown in Figure 17.

30 The human CARD-3 cDNA of Figure 1 (SEQ ID NO:1), which is approximately 1931 nucleotides long including untranslated regions, encodes a protein amino acid having a molecular weight of approximately 61 kDa (excluding post-translational modifications). A plasmid containing  
35 a cDNA encoding human CARD-3 (with the cDNA insert name

- 23 -

of) was deposited with American Type Culture Collection (ATCC), Manassass, VA on \_\_\_\_ and assigned Accession Number \_\_\_\_\_. This deposit will be maintained under the terms of the Budapest Treaty on the International  
5 Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. This deposit was made merely as a convenience for those of skill in the art and is not an admission that a deposit is required under 35 U.S.C. §112.

10           The human CARD-4L cDNA of Figure 3 (SEQ ID NO:7), which is approximately 3382 nucleotides long including untranslated regions, encodes a protein amino acid having a molecular weight of approximately 108 kDa (excluding post-translational modifications). A plasmid containing  
15 a cDNA encoding human CARD-4L (with the cDNA insert name of \_\_\_\_\_) was deposited with American Type Culture Collection (ATCC), Manassass, VA on \_\_\_\_ and assigned Accession Number \_\_\_\_\_. This deposit will be maintained under the terms of the Budapest Treaty on the  
20 International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. This deposit was made merely as a convenience for those of skill in the art and is not an admission that a deposit is required under 35 U.S.C. §112.

25           The human CARD-4S cDNA of Figure 5 (SEQ ID NO:25), which is 3082 nucleotides long including untranslated regions. A plasmid containing a cDNA encoding human CARD-4S (with the cDNA insert name of \_\_\_\_\_) was deposited with American Type Culture Collection (ATCC),  
30 Manassass, VA on \_\_\_\_ and assigned Accession Number \_\_\_\_\_. This deposit will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. This deposit was made merely as a convenience

- 24 -

for those of skill in the art and is not an admission that a deposit is required under 35 U.S.C. §112.

A region of human CARD-4L protein (SEQ ID NO:8) bears some similarity to a CARD domain of CARD-3 (SEQ ID NO:6), ARC-CARD (SEQ ID NO:31), cIAP1-CARD (SEQ ID NO:32), and cIAP2-CARD (SEQ ID NO:33). This comparison is depicted in Figure 7.

Human CARD-3 or CARD-4 are members of a family of molecules (the "CARD family") having certain conserved structural and functional features. The term "family" when referring to the protein and nucleic acid molecules of the invention is intended to mean two or more proteins or nucleic acid molecules having a common structural domain and having sufficient amino acid or nucleotide sequence identity as defined herein. Such family members can be naturally occurring and can be from either the same or different species. For example, a family can contain a first protein of human origin and a homologue of that protein of murine origin, as well as a second, distinct protein of human origin and a murine homologue of that protein. Members of a family may also have common functional characteristics.

In one embodiment, a CARD-3 or CARD-4 protein includes a CARD domain having at least about 65%, preferably at least about 75%, and more preferably about 85%, 95%, or 98% amino acid sequence identity to the CARD domain of SEQ ID NO:6 or the CARD domain of SEQ ID NO:10 or the CARD domain of SEQ ID NO:28.

Preferred CARD-3 or CARD-4 polypeptides of the present invention have an amino acid sequence sufficiently identical to the CARD domain consensus amino acid sequence of SEQ ID NO:6, SEQ ID NO:10, SEQ ID NO:28, respectively. The CARD-3 polypeptide also has an amino acid sequence sufficiently identical to the kinase domain consensus sequence of SEQ ID NO:4, and an amino acid

- 25 -

sequence that is sufficiently identical to the linker domain of SEQ ID NO:5. The CARD-4L polypeptide has an amino acid sequence sufficiently identical to the nucleotide binding domain of SEQ ID NO:11, an amino acid sequence sufficiently identical to the Walker Box "A" of SEQ ID NO:12 or Walker Box "B" of SEQ ID NO:13, an amino acid sequence sufficiently identical to the kinase 1a subdomain of SEQ ID NO:46, an amino acid sequence sufficiently identical to the kinase 2 subdomain of SEQ ID NO:47, or an amino acid sequence sufficiently identical to the kinase 3a subdomain of SEQ ID NO:14, or an amino acid sequence sufficiently identical to the Leucine-rich repeats of SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, and SEQ ID NO:23. As used herein, the term "sufficiently identical" refers to a first amino acid or nucleotide sequence which contains a sufficient or minimum number of identical or equivalent (e.g., an amino acid residue which has a similar side chain) amino acid residues or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences have a common structural domain and/or common functional activity. For example, amino acid or nucleotide sequences which contain a common structural domain having about 65% identity, preferably 75% identity, more preferably 85%, 95%, or 98% identity are defined herein as sufficiently identical.

As used interchangeably herein a "CARD-3 or CARD-4 activity", "biological activity of CARD-3 or CARD-4" or "functional activity of CARD-3 or CARD-4", refers to an activity exerted by a CARD-3 or CARD-4 protein, polypeptide or nucleic acid molecule on a CARD-3 or CARD-4 responsive cell as determined in vivo, or in vitro, according to standard techniques. A CARD-3 or

- 26 -

CARD-4 activity can be a direct activity, such as an association with or an enzymatic activity on a second protein or an indirect activity, such as a cellular signaling activity mediated by interaction of the CARD-3 or CARD-4 protein with a second protein. In an embodiment, a CARD-3 or CARD-4 activity includes at least one or more of the following activities: (i) interaction with proteins in the apoptotic signalling pathway (ii) interaction with a CARD-3 or CARD-4 ligand; or (iii) interaction with an intracellular target protein; (iv) indirect interaction with caspases. For example, in Example 4, CARD-3-containing proteins were shown to associate with CARD-4-containing proteins. In example 9, CARD-4 proteins were shown to induce NF- $\kappa$ B-mediated transcription. In example 10, CARD-3 and CARD-4 were shown to enhance caspase 9 activity.

Accordingly, another embodiment of the invention features isolated CARD-3 or CARD-4 proteins and polypeptides having a CARD-3 or CARD-4 activity.

Various aspects of the invention are described in further detail in the following subsections.

### **I. Isolated Nucleic Acid Molecules**

One aspect of the invention pertains to isolated nucleic acid molecules that encode CARD-3 or CARD-4 proteins or biologically active portions thereof, as well as nucleic acid molecules sufficient for use as hybridization probes to identify CARD-3 or CARD-4-encoding nucleic acids (e.g., CARD-3 or CARD-4 mRNA) and fragments for use as PCR primers for the amplification or mutation of CARD-3 or CARD-4 nucleic acid molecules. As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA) and analogs of the DNA or RNA generated using nucleotide

- 27 -

analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

An "isolated" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated CARD-3 or CARD-4L/S nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

A nucleic acid molecule of the present invention, e.g., a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, the cDNA of ATCC \_\_\_\_\_, the cDNA of ATCC \_\_\_\_\_, or a complement of any of these nucleotide sequences, can be isolated using standard molecular biology techniques and the sequence information provided herein. Using all or portion of the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, the cDNA of ATCC \_\_\_\_\_ or the cDNA of ATCC \_\_\_\_\_, as a hybridization probe, CARD-3 or CARD-4



- 28 -

nucleic acid molecules can be isolated using standard hybridization and cloning techniques (e.g., as described in Sambrook et al., eds., Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

A nucleic acid of the invention can be amplified using cDNA, mRNA or genomic DNA as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to CARD-3 or CARD-4 nucleotide sequences can be prepared by standard synthetic techniques, e.g., using an automated DNA synthesizer.

In another embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule which is a complement of the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, the cDNA of ATCC \_\_\_\_\_ or the cDNA of ATCC \_\_\_\_\_ or a portion thereof. A nucleic acid molecule which is complementary to a given nucleotide sequence is one which is sufficiently complementary to the given nucleotide sequence that it can hybridize to the given nucleotide sequence thereby forming a stable duplex.

Moreover, the nucleic acid molecule of the invention can comprise only a portion of a nucleic acid sequence encoding CARD-3 or CARD-4, for example, a fragment which can be used as a probe or primer or a fragment encoding a biologically active portion of CARD-3 or CARD-4. The nucleotide sequence determined from the cloning of the human CARD-3 or CARD-4, and the partial

- 29 -

murine CARD-4 gene allows for the generation of probes and primers designed for use in identifying and/or cloning CARD-3 or CARD-4 homologues in other cell types, e.g., from other tissues, as well as CARD-3 or CARD-4 homologues and orthologs from other mammals. The probe/primer typically comprises substantially purified oligonucleotide. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12, preferably about 25, more preferably about 50, 75, 100, 125, 150, 175, 200, 250, 300, 350 or 400 consecutive nucleotides of the sense or anti-sense sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, the cDNA of ATCC \_\_\_\_\_, the cDNA of ATCC \_\_\_\_\_ or of a naturally occurring mutant of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, the cDNA of ATCC \_\_\_\_\_, or the cDNA of ATCC \_\_\_\_\_.

Probes based on the human CARD-3 or human CARD-4 or murine CARD-4 nucleotide sequence can be used to detect transcripts or genomic sequences encoding the same or identical proteins. The probe comprises a label group attached thereto, e.g., a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used as a part of a diagnostic test kit for identifying allelic variants and orthologs of the CARD-3 and CARD-4 proteins of the present invention, identifying cells or tissue which mis-express a CARD-3 or CARD-4 protein, such as by measuring a level of a CARD-3 or CARD-4-encoding nucleic acid in a sample of cells from a subject, e.g., detecting CARD-3 or CARD-4 mRNA levels or determining whether a genomic CARD-3 or CARD-4 gene has been mutated or deleted.

A nucleic acid fragment encoding a "biologically active portion of CARD-3 or CARD-4L" can be prepared by isolating a portion of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, or the nucleotide sequence of the cDNA

- 30 -

of ATCC \_\_\_\_\_, or the nucleotide sequence of the cDNA of ATCC \_\_\_\_\_ which encodes a polypeptide having a CARD-3 or CARD-4 biological activity, expressing the encoded portion of CARD-3 or CARD-4 protein (e.g., by recombinant expression in vitro) and assessing the activity of the encoded portion of CARD-3 or CARD-4. For example, a nucleic acid fragment encoding a biologically active portion of CARD-3 or CARD-4 includes a CARD domain, e.g., SEQ ID NO:6 and SEQ ID NO:10 or SEQ ID NO:26.

10 The invention further encompasses nucleic acid molecules that differ from the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, the cDNA of ATCC \_\_\_\_\_ or the cDNA of ATCC \_\_\_\_\_ due to degeneracy of the genetic code and thus encode the same CARD-3 or CARD-4 protein as that encoded by the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, the cDNA of ATCC \_\_\_\_\_ or the cDNA of ATCC \_\_\_\_\_.

20 In addition to the human CARD-3 or CARD-4 nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:38, SEQ ID NO:40, the cDNA of ATCC \_\_\_\_\_, the cDNA of ATCC \_\_\_\_\_, or the cDNA of ATCC \_\_\_\_\_, and the murine CARD-4L cDNA sequence shown in SEQ ID NO:42 it will be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequences of CARD-3 or CARD-4 may exist within a population (e.g., the human population). Such genetic polymorphism in the CARD-3 or CARD-4 gene may exist among individuals within a population due to natural allelic variation. As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules comprising an open reading frame encoding a CARD-3 or

- 31 -

CARD-4 protein, preferably a mammalian CARD-3 or CARD-4 protein. Such natural allelic variations can typically result in 1-5% variance in the nucleotide sequence of the CARD-3 or CARD-4 gene. Any and all such nucleotide variations and resulting amino acid polymorphisms in CARD-3 or CARD-4 that are the result of natural allelic variation and that do not alter the functional activity of CARD-3 or CARD-4 are intended to be within the scope of the invention.

Moreover, nucleic acid molecules encoding CARD-3 or CARD-4 proteins from other species (CARD-3 or CARD-4 orthologs/homologues), which have a nucleotide sequence which differs from that of a human CARD-3 or CARD-4, are intended to be within the scope of the invention. For example, Example 5 describes the murine CARD-4 ortholog. Nucleic acid molecules corresponding to natural allelic variants and homologues of the CARD-3 or CARD-4 cDNA of the invention can be isolated based on their identity to the human CARD-3 or human or murine CARD-4 nucleic acids disclosed herein using the human or murine cDNAs, or a portion thereof, as a hybridization probe according to standard hybridization techniques under stringent hybridization conditions. In general, an allelic variant of a gene will be readily identifiable as mapping to the same chromosomal location as said gene. For example, in Example 6, the chromosomal location of the human CARD-4 gene is discovered to be chromosome 7 close to the SHGC-31928 genetic marker. Allelic variants of human CARD-4 will be readily identifiable as mapping to the human CARD-4 locus on chromosome 7 near genetic marker SHGC-31928.

Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention is at least 300 (325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1300, 1600 or 1931) nucleotides in length

- 32 -

and hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence, preferably the coding sequence, of SEQ ID NO:1, SEQ ID NO:3, or the cDNA of ATCC \_\_\_\_\_. In yet another  
5 embodiment, an isolated nucleic acid molecule of the invention is at least 300 (325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, or 1300, 1640, 1900, 2200, 2500, 2800, 3100, or 3382) nucleotides in length and hybridizes under stringent conditions to the  
10 nucleic acid molecule comprising the nucleotide sequence, preferably the coding sequence, of SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:38, SEQ ID NO:40, or the cDNA of ATCC \_\_\_\_\_. Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention is at least 300  
15 (325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, or 1300, 1640, 1900, 2200, 2500, 2800, 3100, 3300, 3600, 3900, 4200 or 4209) nucleotides in length and hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence,  
20 preferably the coding sequence, of SEQ ID NO:42.

As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 60% (65%, 70%, preferably 75%)  
25 identical to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. An, non-limiting example of stringent  
30 hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C. Preferably, an isolated nucleic acid molecule of the invention that hybridizes under stringent conditions to  
35 the sequence of SEQ ID NO:1, SEQ ID NO:3, the cDNA of

- 33 -

ATCC \_\_\_\_\_ corresponds to a naturally-occurring nucleic acid molecule. As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (e.g.,  
5 encodes a natural protein).

In addition to naturally-occurring allelic variants of the CARD-3 or CARD-4 sequence that may exist in the population, the skilled artisan will further appreciate that changes can be introduced by mutation  
10 into the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, the cDNA of ATCC \_\_\_\_\_, the cDNA of ATCC \_\_\_\_\_, or the cDNA of ATCC \_\_\_\_\_, thereby leading to changes in the amino acid  
15 sequence of the encoded CARD-3, CARD-4L/S protein, CARD-4 splice variant, or murine CARD-4 without altering the functional ability of the CARD-3, CARD-4L/S, CARD-4 splice variant, or murine CARD-4 protein. For example, one can make nucleotide substitutions leading to amino  
20 acid substitutions at "non-essential" amino acid residues. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of CARD-3, CARD-4L/S, CARD-4 splice variant, or murine CARD-4 protein (e.g., the sequence of SEQ ID NO:2, SEQ ID  
25 NO:8, SEQ ID NO:26, SEQ ID NO:39, SEQ ID NO:41 and SEQ ID NO:43) without altering the biological activity, whereas an "essential" amino acid residue is required for biological activity. For example, amino acid residues that are conserved among the CARD-3, CARD-4L/S, CARD-4  
30 splice variant, or murine CARD-4 proteins of various species are predicted to be particularly unamenable to alteration.

For example, preferred CARD-3 or CARD-4 proteins of the present invention, contain at least one CARD  
35 domain. Additionally, a CARD-3 protein also contains at

- 34 -

least one kinase domain or at least one linker domain. A CARD domain contains at least one nucleotide binding domain or Leucine-rich repeats. Such conserved domains are less likely to be amenable to mutation. Other amino acid residues, however, (e.g., those that are not conserved or only semi-conserved among CARD-3 or CARD-4 of various species) may not be essential for activity and thus are likely to be amenable to alteration.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding CARD-3 or CARD-4 proteins that contain changes in amino acid residues that are not essential for activity. Such CARD-3 or CARD-4 proteins differ in amino acid sequence from SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:25, SEQ ID NO:39, SEQ ID NO:41, or SEQ ID NO:43 and yet retain biological activity. In one embodiment, the isolated nucleic acid molecule includes a nucleotide sequence encoding a protein that includes an amino acid sequence that is at least about 45% identical, 65%, 75%, 85%, 95%, or 98% identical to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:26, SEQ ID NO:39, SEQ ID NO:41 or SEQ ID NO:43.

An isolated nucleic acid molecule encoding a CARD-3 or CARD-4 proteins having a sequence which differs from that of SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:26, SEQ ID NO:39, SEQ ID NO:41 or SEQ ID NO:43, can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of CARD-3 (SEQ ID NO:1, SEQ ID NO:3, the cDNA of ATCC \_\_\_\_\_) or CARD-4L (SEQ ID NO:7, SEQ ID NO:9, the cDNA of ATCC \_\_\_\_\_), or CARD-4S (SEQ ID NO:25, SEQ ID NO:27, the cDNA of ATCC \_\_\_\_\_), or human CARD-4 splice variants (SEQ ID NO:38, SEQ ID NO:40, the cDNA of ATCC \_\_\_\_\_ or the cDNA of ATCC \_\_\_\_\_), or murine CARD-4 (SEQ ID NO:42) such that one or more amino acid substitutions, additions or

- 35 -

deletions are introduced into the encoded protein. Mutations can be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid

5 substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having

10 similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine,

15 serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine,

20 phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in CARD-3 or CARD-4 is preferably replaced with another amino acid residue from the same side chain family. Alternatively, mutations can be introduced randomly along all or part of a CARD-3 or

25 CARD-4 coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for CARD-3 or CARD-4 biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed recombinantly and the

30 activity of the protein can be determined.

In an embodiment, a mutant CARD-3 or CARD-4 protein can be assayed for: (1) the ability to form protein:protein interactions with proteins in the apoptotic signalling pathway; (2) the ability to bind a

35 CARD-3 or CARD-4 ligand; or (3) the ability to bind to an



- 36 -

intracellular target protein. For example, (1) in Example 7, a two-hybrid screening assay for the physical interaction of CARD-3 and CARD-4 is shown, (2) in Example 8, a two-hybrid system assay for the interaction between CARD-4 and its ligand hNUDC is described, and (3) in Example 12, a coimmunoprecipitation assay for the interaction of CARD-3 with its ligand CARD-4 is shown. In yet another embodiment, a mutant CARD-3 or CARD-4 protein can be assayed for the ability to modulate cellular proliferation, cellular differentiation, or cellular death. For example, in Example 10, assays for the regulation of cellular death (apoptosis) by CARD-3 or CARD-4 are described. In yet another embodiment, a mutant CARD-3 or CARD-4 protein can be assayed for regulation of a cellular signal transduction pathway. For example, in Example 9, an assay for the regulation by CARD-4 of the NF- $\kappa$ B pathway is described.

The present invention encompasses antisense nucleic acid molecules, i.e., molecules which are complementary to a sense nucleic acid encoding a protein, e.g., complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense nucleic acid can be complementary to an entire CARD-3 or CARD-4 coding strand, or to only a portion thereof, e.g., all or part of the protein coding region (or open reading frame). An antisense nucleic acid molecule can be antisense to a noncoding region of the coding strand of a nucleotide sequence encoding CARD-3 or CARD-4. The noncoding regions ("5' and 3' untranslated regions") are the 5' and 3' sequences which flank the coding region and are not translated into amino acids.

Given the coding strand sequences encoding CARD-3 or CARD-4 disclosed herein (e.g., SEQ ID NO:1, SEQ ID

- 37 -

NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:38, SEQ ID NO:40, or SEQ ID NO:42), antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing.

5 The antisense nucleic acid molecule can be complementary to the entire coding region of CARD-3 or CARD-4L/S mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of CARD-3 or CARD-4 mRNA. For example, the

10 antisense oligonucleotide can be complementary to the region surrounding the translation start site of CARD-3 mRNA, e.g., an oligonucleotide having the sequence CCCTGGTACTTGCCCCTCCGGTAG (SEQ ID NO:34) or CCTGGTACTTGCCCCTCC (SEQ ID NO:35) or of the CARD-4L mRNA

15 e.g., TCGTTAAGCCCTTGAAGACAGTG (SEQ ID NO:36) and TCGTTAGCCCTTGAAGACCAAGTGAGTGTAG (SEQ ID NO:37). An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can

20 be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously

25 modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used.

30 Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil,

35 5-carboxymethylaminomethyl-2-thiouridine,

- 38 -

5-carboxymethylaminomethyluracil, dihydrouracil,  
beta-D-galactosylqueosine, inosine,  
N6-isopentenyladenine, 1-methylguanine, 1-methylinosine,  
2,2-dimethylguanine, 2-methyladenine, 2-methylguanine,  
5 3-methylcytosine, 5-methylcytosine, N6-adenine,  
7-methylguanine, 5-methylaminomethyluracil,  
5-methoxyaminomethyl-2-thiouracil,  
beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil,  
5-methoxyuracil, 2-methylthio-N6-isopentenyladenine,  
10 uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil,  
queosine, 2-thiocytosine, 5-methyl-2-thiouracil,  
2-thiouracil, 4-thiouracil, 5-methyluracil,  
uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic  
acid (v), 5-methyl-2-thiouracil,  
15 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and  
2,6-diaminopurine. Alternatively, the antisense nucleic  
acid can be produced biologically using an expression  
vector into which a nucleic acid has been subcloned in an  
antisense orientation (i.e., RNA transcribed from the  
20 inserted nucleic acid will be of an antisense orientation  
to a target nucleic acid of interest, described further  
in the following subsection).

The antisense nucleic acid molecules of the  
invention are typically administered to a subject or  
25 generated in situ such that they hybridize with or bind  
to cellular mRNA and/or genomic DNA encoding a CARD-3 or  
CARD-4 protein to thereby inhibit expression of the  
protein, e.g., by inhibiting transcription and/or  
translation. The hybridization can be by conventional  
30 nucleotide complementarity to form a stable duplex, or,  
for example, in the case of an antisense nucleic acid  
molecule which binds to DNA duplexes, through specific  
interactions in the major groove of the double helix. An  
example of a route of administration of antisense nucleic  
35 acid molecules of the invention include direct injection

- 39 -

at a tissue site. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, e.g., by linking the antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

An antisense nucleic acid molecule of the invention can be an  $\alpha$ -anomeric nucleic acid molecule. An  $\alpha$ -anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual  $\beta$ -units, the strands run parallel to each other (Gaultier et al. (1987) Nucleic Acids. Res. 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-O-methylribonucleotide (Inoue et al. (1987) Nucleic Acids Res. 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue et al. (1987) FEBS Lett. 215:327-330).

The invention also encompasses ribozymes. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (e.g., hammerhead ribozymes (described in Haselhoff and Gerlach (1988) Nature 334:585-591)) can be used to catalytically cleave CARD-3 or CARD-4 mRNA transcripts to thereby inhibit translation of CARD-3 or CARD-4 mRNA. A ribozyme having

- 40 -

specificity for a CARD-3 or CARD-4-encoding nucleic acid can be designed based upon the nucleotide sequence of a CARD-3 or CARD-4 cDNA disclosed herein (e.g., SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:38, SEQ ID NO:40, and SEQ ID NO:42). For example, a derivative of a Tetrahymena L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in a CARD-3 or CARD-4-encoding mRNA. See, e.g., Cech et al. U.S. Patent No. 4,987,071; and Cech et al. U.S. Patent No. 5,116,742. Alternatively, CARD-3 or CARD-4 mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, e.g., Bartel and Szostak (1993) Science 261:1411-1418.

The invention also encompasses nucleic acid molecules which form triple helical structures. For example, CARD-3 or CARD-4 gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the CARD-3 or CARD-4 (e.g., the CARD-3 or CARD-4 promoter and/or enhancers) to form triple helical structures that prevent transcription of the CARD-3 or CARD-4 gene in target cells. See generally, Helene (1991) Anticancer Drug Des. 6(6):569-84; Helene (1992) Ann. N.Y. Acad. Sci. 660:27-36; and Maher (1992) Bioassays 14(12):807-15.

In embodiments, the nucleic acid molecules of the invention can be modified at the base moiety, sugar moiety or phosphate backbone to improve, e.g., the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acids can be modified to generate peptide nucleic acids (see Hyrup et al. (1996) Bioorganic & Medicinal Chemistry 4(1): 5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid

- 41 -

mimics, e.g., DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to  
5 allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup et al. (1996) supra; Perry-O'Keefe et al. (1996) Proc. Natl.  
10 Acad. Sci. USA 93: 14670-675.

PNAs of CARD-3 or CARD-4 can be used for therapeutic and diagnostic applications. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, e.g.,  
15 inducing transcription or translation arrest or inhibiting replication. PNAs of CARD-3 or CARD-4 can also be used, e.g., in the analysis of single base pair mutations in a gene by, e.g., PNA directed PCR clamping; as 'artificial restriction enzymes when used in  
20 combination with other enzymes, e.g., S1 nucleases (Hyrup (1996) supra; or as probes or primers for DNA sequence and hybridization (Hyrup (1996) supra; Perry-O'Keefe et al. (1996) Proc. Natl. Acad. Sci. USA 93: 14670-675).

In another embodiment, PNAs of CARD-3 or CARD-4  
25 can be modified, e.g., to enhance their stability or cellular uptake, by attaching lipophilic or other helper groups to PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques of drug delivery known in the art. For example, PNA-DNA chimeras  
30 of CARD-3 or CARD-4 can be generated which may combine the advantageous properties of PNA and DNA. Such chimeras allow DNA recognition enzymes, e.g., RNase H and DNA polymerases, to interact with the DNA portion while the PNA portion would provide high binding affinity and  
35 specificity. PNA-DNA chimeras can be linked using

- 42 -

linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup (1996) *supra*). The synthesis of PNA-DNA chimeras can be performed as described in Hyrup  
5 (1996) *supra* and Finn et al. (1996) *Nucleic Acids Research* 24(17):3357-63. For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry and modified nucleoside analogs, e.g.,  
10 5'-(4-methoxytrityl)amino-5'-deoxy-thymidine phosphoramidite, can be used as a between the PNA and the 5' end of DNA (Mag et al. (1989) *Nucleic Acid Res.* 17:5973-88). PNA monomers are then coupled in a stepwise manner to produce a chimeric molecule with a 5' PNA  
15 segment and a 3' DNA segment (Finn et al. (1996) *Nucleic Acids Research* 24(17):3357-63). Alternatively, chimeric molecules can be synthesized with a 5' DNA segment and a 3' PNA segment (Peterser et al. (1975) *Bioorganic Med. Chem. Lett.* 5:1119-11124).

20 In other embodiments, the oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, e.g., Letsinger et al. (1989) *Proc. Natl. Acad. Sci. USA*  
25 86:6553-6556; Lemaitre et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:648-652; PCT Publication No. W0 88/09810) or the blood-brain barrier (see, e.g., PCT Publication No. W0 89/10134). In addition, oligonucleotides can be modified with hybridization-triggered cleavage agents  
30 (see, e.g., Krol et al. (1988) *Bio/Techniques* 6:958-976) or intercalating agents (see, e.g., Zon (1988) *Pharm. Res.* 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport  
35 agent, hybridization-triggered cleavage agent, etc.

- 43 -

## II. Isolated CARD-3 or CARD-4 Proteins and Anti-CARD-3 or CARD-4 Antibodies.

One aspect of the invention pertains to isolated CARD-3 or CARD-4 proteins, and biologically active portions thereof, as well as polypeptide fragments suitable for use as immunogens to raise anti-CARD-3 or CARD-4 antibodies. In one embodiment, native CARD-3 or CARD-4 proteins can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. In another embodiment, CARD-3 or CARD-4 proteins are produced by recombinant DNA techniques. Alternative to recombinant expression, a CARD-3 or CARD-4 protein or polypeptide can be synthesized chemically using standard peptide synthesis techniques.

An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the CARD-3 or CARD-4 protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of CARD-3 or CARD-4 protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. Thus, CARD-3 or CARD-4 protein that is substantially free of cellular material includes preparations of CARD-3 or CARD-4 protein having less than about 30%, 20%, 10%, or 5% (by dry weight) of non-CARD-3 or CARD-4 protein (also referred to herein as a "contaminating protein"). When the CARD-3 or CARD-4 protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20%, 10%, or 5%



- 44 -

of the volume of the protein preparation. When CARD-3 or CARD-4 protein is produced by chemical synthesis, it is preferably substantially free of chemical precursors or other chemicals, i.e., it is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. Accordingly such preparations of CARD-3 or CARD-4 protein have less than about 30%, 20%, 10%, 5% (by dry weight) of chemical precursors or non-CARD-3 or CARD-4 chemicals.

Biologically active portions of a CARD-3 or CARD-4 protein include peptides comprising amino acid sequences sufficiently identical to or derived from the amino acid sequence of the CARD-3 or CARD-4 protein (e.g., the amino acid sequence shown in SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:26, SEQ ID NO:39, SEQ ID NO:41 or SEQ ID NO:43), which include less amino acids than the full length CARD-3 or CARD-4 proteins, and exhibit at least one activity of a CARD-3 or CARD-4 protein. Typically, biologically active portions comprise a domain or motif with at least one activity of the CARD-3 or CARD-4 protein. A biologically active portion of a CARD-3 or CARD-4 protein can be a polypeptide which is, for example, 10, 25, 50, 100 or more amino acids in length. Preferred biologically active polypeptides include one or more identified CARD-3 or CARD-4 structural domains, e.g., the CARD domain (SEQ ID NO:6 or SEQ ID NO:10 or SEQ ID NO:27).

Moreover, other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the functional activities of a native CARD-3 or CARD-4 protein.

CARD-3 or CARD-4 protein has the amino acid sequence shown of SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:26, SEQ ID NO:39, SEQ ID NO:41 or SEQ ID NO:43. Other useful CARD-3 or CARD-4 proteins are substantially identical to

- 45 -

SEQ ID NO:2 or SEQ ID NO:8 or SEQ ID NO:26, SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID NO:43 and retain the functional activity of the protein of SEQ ID NO:2 or SEQ ID NO:8 or SEQ ID NO:26, SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID NO:43 yet differ in amino acid sequence due to natural allelic variation or mutagenesis. CARD-3 and CARD-4 are involved in activating caspases in the apoptotic pathway. For example, in Example 10, CARD-4 is shown to enhance caspase 9 activity. Accordingly, a useful CARD-3 or CARD-4 protein is a protein which includes an amino acid sequence at least about 45%, preferably 55%, 65%, 75%, 85%, 95%, or 99% identical to the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:8 or SEQ ID NO:26, SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID NO:43 and retains the functional activity of the CARD-3 or CARD-4 proteins of SEQ ID NO:2 or SEQ ID NO:8 or SEQ ID NO:26, SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID NO:43. In other instances, the CARD-3 or CARD-4 protein is a protein having an amino acid sequence 55%, 65%, 75%, 85%, 95%, or 98% identical to the CARD-3 or CARD-4L CARD domain (SEQ ID NO:6, SEQ ID NO:10 and SEQ ID NO:27). In an embodiment, the CARD-3 or CARD-4 protein retains a functional activity of the CARD-3 or CARD-4 protein of SEQ ID NO:2, SEQ ID NO:8 or SEQ ID NO:26, SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID NO:43.

To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in

- 46 -

the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = #  
5 of identical positions/total # of positions x 100).

The determination of percent homology between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two  
10 sequences is the algorithm of Karlin and Altschul (1990) Proc. Nat'l Acad. Sci. USA 87:2264-2268, modified as in Karlin and Altschul (1993) Proc. Nat'l Acad. Sci. USA 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al. (1990) J.  
15 Mol. Biol. 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences similar or homologous to CARD-3 or CARD-4 nucleic acid molecules of the invention. For example, Example 5 describes the use  
20 of the TBLASTN program to query a database of sequences of full length and partial cDNA sequences with the human CARD-4 polypeptide sequence leading to the discovery of murine CARD-4 and Example 4 describes the use of BLASTN to query a proprietary EST database with the 5'  
25 untranslated sequence of CARD-4 leading to the discovery of two human CARD-4 splice variants. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to CARD-3 or CARD-4 protein molecules of the  
30 invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g.,  
35 XBLAST and NBLAST) can be used. See

- 47 -

<http://www.ncbi.nlm.nih.gov>. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is

5 incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

10           The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, only exact matches are counted.

          The invention also provides CARD-3 or CARD-4

15 chimeric or fusion proteins. As used herein, a CARD-3 or CARD-4 "chimeric protein" or "fusion protein" comprises a CARD-3 or CARD-4 polypeptide operatively linked to a non-CARD-3 or CARD-4 polypeptide. A "CARD-3 or CARD-4 polypeptide" refers to a polypeptide having an amino acid

20 sequence corresponding to CARD-3 or CARD-4L/S, murine CARD-4 or human CARD-4 splice variants, whereas a "non-CARD-3 or CARD-4 polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially identical to the

25 CARD-3 or CARD-4L/S protein, murine CARD-4, or human CARD-4 splice variants e.g., a protein which is different from the CARD-3 or CARD-4 proteins and which is derived from the same or a different organism. Within a CARD-3 or CARD-4L fusion protein, the CARD-3 or CARD-4

30 polypeptide can correspond to all or a portion of a CARD-3 or CARD-4 protein, preferably at least one biologically active portion of a CARD-3 or CARD-4 protein. Within the fusion protein, the term "operatively linked" is intended to indicate that the

35 CARD-3 or CARD-4 polypeptide and the non-CARD-3 or

- 48 -

non-CARD-4 polypeptide are fused in-frame to each other. The non-CARD-3 or non-CARD-4 polypeptide can be fused to the N-terminus or C-terminus of the CARD-3 or CARD-4 polypeptide.

5           One useful fusion protein is a GST-CARD-3 or GST-CARD-4 fusion protein in which the CARD-3 or CARD-4 sequences are fused to the C-terminus of the GST sequences. Such fusion proteins can facilitate the purification of recombinant CARD-3 or CARD-4.

10           In another embodiment, the fusion protein contains a signal sequence from another protein. In certain host cells (e.g., mammalian host cells), expression and/or secretion of CARD-3 or CARD-4 can be increased through use of a heterologous signal sequence. For example, the  
15 gp67 secretory sequence of the baculovirus envelope protein can be used as a heterologous signal sequence (Current Protocols in Molecular Biology, Ausubel et al., eds., John Wiley & Sons, 1992). Other examples of eukaryotic heterologous signal sequences include the  
20 secretory sequences of melittin and human placental alkaline phosphatase (Stratagene; La Jolla, California). In yet another example, useful prokaryotic heterologous signal sequences include the phoA secretory signal (Molecular cloning, Sambrook et al, second edition, Cold  
25 spring harbor laboratory press, 1989) and the protein A secretory signal (Pharmacia Biotech; Piscataway, New Jersey).

          In yet another embodiment, the fusion protein is a CARD-3 or CARD-4-immunoglobulin fusion protein in which  
30 all or part of CARD-3 or CARD-4 is fused to sequences derived from a member of the immunoglobulin protein family. The CARD-3 or CARD-4-immunoglobulin fusion proteins of the invention can be incorporated into pharmaceutical compositions and administered to a subject  
35 to inhibit an interaction between a CARD-3 or CARD-4

- 49 -

ligand and a CARD-3 or CARD-4 protein on the surface of a cell, to thereby suppress CARD-3 or CARD-4-mediated signal transduction in vivo. The CARD-3 or CARD-4-immunoglobulin fusion proteins can be used to  
5 affect the bioavailability of a CARD-3 or CARD-4 cognate ligand. Inhibition of the CARD-3 ligand/CARD-3 or CARD-4 ligand/CARD-4 interaction may be useful therapeutically for both the treatment of proliferative and  
10 differentiative disorders, as well as modulating (e.g. promoting or inhibiting) cell survival. Moreover, the CARD-3 or CARD-4-immunoglobulin fusion proteins of the invention can be used as immunogens to produce anti-CARD-3 or CARD-4 antibodies in a subject, to purify CARD-3 or CARD-4 ligands and in screening assays to  
15 identify molecules which inhibit the interaction of CARD-3 or CARD-4 with a CARD-3 or CARD-4 ligand.

Preferably, a CARD-3 or CARD-4 chimeric or fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments  
20 coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in  
25 of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR  
30 amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, e.g., Current Protocols in  
35 Molecular Biology, Ausubel et al. eds., John Wiley &

- 50 -

Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (e.g., a GST polypeptide). A CARD-3 or CARD-4-encoding nucleic acid can be cloned into such an  
5 expression vector such that the fusion moiety is linked in-frame to the CARD-3 or CARD-4 protein.

The present invention also pertains to variants of the CARD-3 or CARD-4 proteins which function as either CARD-3 or CARD-4 agonists (mimetics) or as CARD-3 or  
10 CARD-4 antagonists. Variants of the CARD-3 or CARD-4 protein can be generated by mutagenesis, e.g., discrete point mutation or truncation of the CARD-3 or CARD-4 protein. An agonist of the CARD-3 or CARD-4 protein can retain substantially the same, or a subset, of the  
15 biological activities of the naturally occurring form of the CARD-3 or CARD-4 protein. An antagonist of the CARD-3 or CARD-4 protein can inhibit one or more of the activities of the naturally occurring form of the CARD-3 or CARD-4 protein by, for example, competitively binding  
20 to a downstream or upstream member of a cellular signaling cascade which includes the CARD-3 or CARD-4 protein. Thus, specific biological effects can be elicited by treatment with a variant of limited function. Treatment of a subject with a variant having a subset of  
25 the biological activities of the naturally occurring form of the protein can have fewer side effects in a subject relative to treatment with the naturally occurring form of the CARD-3 or CARD-4 proteins.

Variants of the CARD-3 or CARD-4 protein which  
30 function as either CARD-3 or CARD-4 agonists (mimetics) or as CARD-3 or CARD-4 antagonists can be identified by screening combinatorial libraries of mutants, e.g., truncation mutants of the CARD-3 or CARD-4 protein for CARD-3 or CARD-4 protein agonist or antagonist activity.  
35 In one embodiment, a variegated library of CARD-3 or

- 51 -

CARD-4 variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of CARD-3 or CARD-4 variants can be produced by, for example, enzymatically  
5 ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential CARD-3 or CARD-4 sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display) containing the set of  
10 CARD-3 or CARD-4 sequences therein. There are a variety of methods which can be used to produce libraries of potential CARD-3 or CARD-4 variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic  
15 DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential CARD-3 or CARD-4 sequences. Methods for  
20 synthesizing degenerate oligonucleotides are known in the art (see, e.g., Narang (1983) Tetrahedron 39:3; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477).

25           Useful fragments of CARD-3 and CARD-4 include fragments comprising or consisting of a domain or subdomain described herein, e.g., a kinase domain or a CARD domain.

          In addition, libraries of fragments of the CARD-3  
30 or CARD-4 protein coding sequence can be used to generate a variegated population of CARD-3 or CARD-4 fragments for screening and subsequent selection of variants of a CARD-3 or CARD-4 protein. In one embodiment, a library of coding sequence fragments can be generated by treating  
35 a double stranded PCR fragment of a CARD-3 or CARD-4



- 52 -

coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense  
5 pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes  
10 N-terminal and internal fragments of various sizes of the CARD-3 or CARD-4 protein.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA  
15 libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of CARD-3 or CARD-4 proteins. The most widely used techniques, which are amenable to high through-put  
20 analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection  
25 of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to  
30 identify CARD-3 or CARD-4 variants (Arkin and Yourvan (1992) Proc. Natl. Acad. Sci. USA 89:7811-7815; Delgrave et al. (1993) Protein Engineering 6(3):327-331).

An isolated CARD-3 or CARD-4 protein, or a portion or fragment thereof, can be used as an immunogen to  
35 generate antibodies that bind CARD-3 or CARD-4 using

- 53 -

standard techniques for polyclonal and monoclonal antibody preparation. The full-length CARD-3 or CARD-4 protein can be used or, alternatively, the invention provides antigenic peptide fragments of CARD-3 or CARD-4 for use as immunogens. The antigenic peptide of CARD-3 or CARD-4 comprises at least 8 (preferably 10, 15, 20, or 30) amino acid residues of the amino acid sequence shown in SEQ ID NO:2, SEQ ID NO:8 or SEQ ID NO:26, or SEQ ID NO:39 or SEQ ID NO:41 or SEQ ID NO:43 or polypeptides including amino acids 128-139 or 287-298 of human CARD-4L and encompasses an epitope of CARD-3 or CARD-4 such that an antibody raised against the peptide forms a specific immune complex with CARD-3 or CARD-4.

Useful antibodies include antibodies which bind to a domain or subdomain of CARD-3 or CARD-4 described herein, e.g., a kinase domain or a CARD domain).

Preferred epitopes encompassed by the antigenic peptide are regions of CARD-3 or CARD-4 that are located on the surface of the protein, e.g., hydrophilic regions. Other important criteria include a preference for a terminal sequence, high antigenic index (e.g., as predicted by Jameson-Wolf algorithm), ease of peptide synthesis (e.g., avoidance of prolines); and high surface probability (e.g., as predicted by the Emini algorithm; Figure 8 and Figure 9).

A CARD-3 or CARD-4 immunogen typically is used to prepare antibodies by immunizing a suitable subject, (e.g., rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, recombinantly expressed CARD-3 or CARD-4 protein or a chemically synthesized CARD-3 or CARD-4 polypeptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic

- 54 -

CARD-3 or CARD-4 preparation induces a polyclonal anti-CARD-3 or CARD-4 antibody response. For example, polypeptides including amino acids 128-139 or 287-298 of human CARD-4L were conjugated to KLH and the resulting  
5 conjugates were used to immunize rabbits and polyclonal antibodies that specifically recognize the two immunogen peptides were generated.

Accordingly, another aspect of the invention pertains to anti-CARD-3 or CARD-4 antibodies. The term  
10 "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically binds an antigen, such as CARD-3 or CARD-4. A molecule which specifically  
15 binds to CARD-3 or CARD-4 is a molecule which binds CARD-3 or CARD-4, but does not substantially bind other molecules in a sample, e.g., a biological sample, which naturally contains CARD-3 or CARD-4. Examples of immunologically active portions of immunoglobulin  
20 molecules include F(ab) and F(ab')<sub>2</sub> fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies that bind CARD-3 or CARD-4. The term "monoclonal antibody" or "monoclonal antibody  
25 composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of CARD-3 or CARD-4. A monoclonal antibody composition thus typically displays a single  
30 binding affinity for a particular CARD-3 or CARD-4 protein with which it immunoreacts.

Polyclonal anti-CARD-3 or CARD-4 antibodies can be prepared as described above by immunizing a suitable subject with a CARD-3 or CARD-4 immunogen. The  
35 anti-CARD-3 or CARD-4 antibody titer in the immunized

- 55 -

subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized CARD-3 or CARD-4. If desired, the antibody molecules directed against CARD-3 or CARD-4 can be isolated from the mammal (e.g., from the blood) and further purified by well-known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, e.g., when the anti-CARD-3 or CARD-4 antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497, the human B cell hybridoma technique (Kozbor et al. (1983) *Immunol Today* 4:72), the EBV-hybridoma technique (Cole et al. (1985), *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing various antibodies monoclonal antibody hybridomas is well known (see generally *Current Protocols in Immunology* (1994) Coligan et al. (eds.) John Wiley & Sons, Inc., New York, NY). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a CARD-3 or CARD-4 immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds CARD-3 or CARD-4.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-CARD-3 or CARD-4 monoclonal antibody (see, e.g., *Current Protocols in Immunology*, supra; Galfre et al. (1977) *Nature* 266:55052; R.H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing

- 56 -

Corp., New York, New York (1980); and Lerner (1981) Yale J. Biol. Med., 54:387-402. Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods which also would be useful.

5 Typically, the immortal cell line (e.g., a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an

10 immortalized mouse cell line, e.g., a myeloma cell line that is sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, e.g.,

15 the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from ATCC. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then

20 selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture

25 supernatants for antibodies that bind CARD-3 or CARD-4, e.g., using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-CARD-3 or CARD-4 antibody can be identified and isolated by

30 screening a recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library) with CARD-3 or CARD-4 to thereby isolate immunoglobulin library members that bind CARD-3 or CARD-4. Kits for generating and screening phage display libraries are

35 commercially available (e.g., the Pharmacia Recombinant

- 57 -

Phage Antibody System, Catalog No. 27-9400-01; and the Stratagene SurfZAP Phage Display Kit, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, U.S. Patent No. 5,223,409; PCT Publication No. WO 92/18619; PCT Publication No. WO 91/17271; PCT Publication No. WO 92/20791; PCT Publication No. WO 92/15679; PCT Publication No. WO 93/01288; PCT Publication No. WO 92/01047; PCT Publication No. WO 92/09690; PCT Publication No. WO 90/02809; Fuchs et al. (1991) Bio/Technology 9:1370-1372; Hay et al. (1992) Hum. Antibod. Hybridomas 3:81-85; Huse et al. (1989) Science 246:1275-1281; Griffiths et al. (1993) EMBO J 12:725-734.

Additionally, recombinant anti-CARD-3 or CARD-4 antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in PCT Publication No. WO 87/02671; European Patent Application 184,187; European Patent Application 171,496; European Patent Application 173,494; PCT Publication No. WO 86/01533; U.S. Patent No. 4,816,567; European Patent Application 125,023; Better et al. (1988) Science 240:1041-1043; Liu et al. (1987) Proc. Natl. Acad. Sci. USA 84:3439-3443; Liu et al. (1987) J. Immunol. 139:3521-3526; Sun et al. (1987) Proc. Natl. Acad. Sci. USA 84:214-218; Nishimura et al. (1987) Canc. Res. 47:999-1005; Wood et al. (1985) Nature 314:446-449; and Shaw et al. (1988) J. Natl. Cancer Inst. 80:1553-1559; Morrison, (1985) Science 229:1202-1207; Oi et al. (1986) Bio/Techniques 4:214; U.S. Patent 5,225,539; Jones et al. (1986) Nature

- 58 -

321:552-525; Verhoeyan et al. (1988) Science 239:1534; and Beidler et al. (1988) J. Immunol. 141:4053-4060.

An anti-CARD-3 or CARD-4 antibody (e.g., monoclonal antibody) can be used to isolate CARD-3 or  
5 CARD-4 by standard techniques, such as affinity chromatography or immunoprecipitation. An anti-CARD-3 or CARD-4 antibody can facilitate the purification of natural CARD-3 or CARD-4 from cells and of recombinantly produced CARD-3 or CARD-4 expressed in host cells.  
10 Moreover, an anti-CARD-3 or CARD-4 antibody can be used to detect CARD-3 or CARD-4 protein (e.g., in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the CARD-3 or CARD-4 protein. Anti-CARD-3 or CARD-4 antibodies can be  
15 used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, e.g., to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of  
20 detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase,  $\beta$ -galactosidase, or  
25 acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine  
30 fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$  or  $^3\text{H}$ .

- 59 -

### III. Recombinant Expression Vectors and Host Cells

Another aspect of the invention pertains to vectors, preferably expression vectors, containing a nucleic acid encoding CARD-3 or CARD-4 (or a portion thereof). As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors, expression vectors, are capable of directing the expression of genes to which they are operatively linked. In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids (vectors). However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic



- 60 -

acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for  
5 expression of the nucleotide sequence (e.g., in an in vitro transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to include promoters, enhancers and other expression control  
10 elements (e.g., polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct  
15 constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design  
20 of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including  
25 fusion proteins or peptides, encoded by nucleic acids as described herein (e.g., CARD-3 or CARD-4 proteins, mutant forms of CARD-3 or CARD-4, fusion proteins, etc.).

The recombinant expression vectors of the invention can be designed for expression of CARD-3 or  
30 CARD-4 in prokaryotic or eukaryotic cells, e.g., bacterial cells such as E. coli, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, Gene Expression Technology: Methods in  
35 Enzymology 185, Academic Press, San Diego, CA (1990).

- 61 -

Alternatively, the recombinant expression vector can be transcribed and translated in vitro, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith and Johnson (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann et al., (1988) *Gene* 69:301-315) and pET 11d (Studier et al., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac

- 62 -

fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident ? prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada et al. (1992) Nucleic Acids Res. 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the CARD-3 or CARD-4 expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari et al. (1987) EMBO J. 6:229-234), pMFa (Kurjan and Herskowitz, (1982) Cell 30:933-943), pJRY88 (Schultz et al. (1987) Gene 54:113-123), pYES2 (Invitrogen Corporation, San Diego, CA), pGBT9 (Clontech, Palo Alto, CA), pGAD10 (Clontech, Palo Alto, CA), pYADE4 and pYGAE2 and pYPGE2 (Brunelli and Pall, (1993) Yeast 9:1299-1308), pYPGE15 (Brunelli and Pall, (1993) Yeast 9:1309-1318), pACTII (Dr. S.E. Elledge, Baylor College of Medicine), and picZ (Invitrogen Corp, San Diego, CA). For example, in Example 7 the expression of a fusion protein comprising amino acids 1-145 of human CARD-4L fused to the DNA-binding domain of *S. cerevisiae*

- 63 -

transcription factor GAL4 from the yeast expression vector pGBT9 is described. In another example, Example 8 describes the expression of a fusion protein comprising amino acids 406-953 of human CARD-4L fused to the DNA-binding domain of *S. cerevisiae* transcription factor GAL4 from the yeast expression vector pGBT9. In yet another example, Example 7 describes the expression of a fusion protein comprising CARD-3 fused to the transcriptional activation domain of *S. cerevisiae* transcription factor GAL4 from the yeast expression vector pACTII.

Alternatively, CARD-3 or CARD-4 can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith et al. (1983) Mol. Cell Biol. 3:2156-2165) and the pVL series (Lucklow and Summers (1989) Virology 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed (1987) Nature 329:840), pCI (Promega), and PMT2PC (Kaufman et al. (1987) EMBO J. 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook et al. (supra). For example, Example 9, Example 10, and Example 12 describe the expression of human CARD-4 or fragments thereof, CARD-3, or both from the mammalian expression vector pCI.

- 64 -

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert et al. (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji et al. (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund et al. (1985) *Science* 230:912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the  $\alpha$ -fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to CARD-3 or CARD-4 mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the

- 65 -

antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense  
5 RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into  
10 which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub et al. (Reviews - Trends in Genetics, Vol. 1(1) 1986).

Another aspect of the invention pertains to host  
15 cells into which a recombinant expression vector of the invention or isolated nucleic acid molecule of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the  
20 particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are  
25 still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, CARD-3 or CARD-4 protein can be expressed in bacterial cells such as E. coli, insect  
30 cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art. For example, in Example 7 a *Saccharomyces cerevisiae* host cell for recombinant CARD-4 and CARD-3 expression is  
35 described and in Examples 9, 10, and 12 293T host cells

- 66 -

for expression of CARD4 or fragments thereof or CARD-3 are described.

Vector DNA or an isolated nucleic acid molecule of the invention can be introduced into prokaryotic or  
5 eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell,  
10 including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, et al. (supra), and other laboratory manuals.

15 For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. IN some cases vector DNA is retained by the host cell.  
20 In other cases the host cell does not retain vector DNA and retains only an isolated nucleic acid molecule of the invention carried by the vector. In some cases, and isolated nucleic acid molecule of the invention is used to transform a cell without the use of a vector.

25 In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance  
30 to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding CARD-3 or CARD-4 or can be introduced on a separate vector. Cells stably transfected with the  
35 introduced nucleic acid can be identified by drug

- 67 -

selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a  
5 prokaryotic or eukaryotic host cell in culture, can be used to produce a (i.e., express) CARD-3 or CARD-4 protein. Accordingly, the invention further provides methods for producing CARD-3 or CARD-4 protein using the host cells of the invention. In one embodiment, the  
10 method comprises culturing the host cell of the invention (into which a recombinant expression vector or isolated nucleic acid molecule encoding CARD-3 or CARD-4 has been introduced) in a suitable medium such that CARD-3 or CARD-4 protein is produced. In another embodiment, the  
15 method further comprises isolating CARD-3 or CARD-4 from the medium or the host cell.

The host cells of the invention can also be used to produce nonhuman transgenic animals. For example, in one embodiment, a host cell of the invention is a  
20 fertilized oocyte or an embryonic stem cell into which CARD-3 or CARD-4-coding sequences have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous CARD-3 or CARD-4 sequences have been introduced into their genome or  
25 homologous recombinant animals in which endogenous CARD-3 or CARD-4 sequences have been altered. Such animals are useful for studying the function and/or activity of CARD-3 or CARD-4 and for identifying and/or evaluating modulators of CARD-3 or CARD-4 activity. As used herein,  
30 a "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats,  
35 chickens, amphibians, etc. A transgene is exogenous DNA



- 68 -

which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell  
5 types or tissues of the transgenic animal. As used herein, an "homologous recombinant animal" is a non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous CARD-3 or CARD-4 gene has been altered by homologous recombination between the  
10 endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, e.g., an embryonic cell of the animal, prior to development of the animal.

A transgenic animal of the invention can be created by introducing CARD-3 or CARD-4-encoding nucleic  
15 acid into the male pronuclei of a fertilized oocyte, e.g., by microinjection, retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. The CARD-3 or CARD-4 cDNA sequence e.g., that of (SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID  
20 NO:9, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42 or the cDNA of ATCC \_\_\_\_\_, or the cDNA of ATCC \_\_\_\_\_, or the cDNA of ATCC \_\_\_\_\_) can be introduced as a transgene into the genome of a non-human animal. Alternatively, a nonhuman homolog or ortholog of  
25 the human CARD-3 or CARD-4 gene, such as a mouse CARD-3 or CARD-4 gene, can be isolated based on hybridization to the human CARD-3 or CARD-4 cDNA and used as a transgene. For example, the mouse ortholog of CARD-4, Figure 15 and SEQ ID NO:42 can be used to make a transgenic animal  
30 using standard methods. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to the CARD-3 or CARD-4 transgene to  
35 direct expression of CARD-3 or CARD-4 protein to

- 69 -

particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, 5 in U.S. Patent Nos. 4,736,866 and 4,870,009, U.S. Patent No. 4,873,191 and in Hogan, Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic 10 founder animal can be identified based upon the presence of the CARD-3 or CARD-4 transgene in its genome and/or expression of CARD-3 or CARD-4 mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. 15 Moreover, transgenic animals carrying a transgene encoding CARD-3 or CARD-4 can further be bred to other transgenic animals carrying other transgenes.

To create an homologous recombinant animal, a vector is prepared which contains at least a portion of a 20 CARD-3 or CARD-4 gene (e.g., a human or a non-human homolog of the CARD-3 or CARD-4 gene, e.g., a murine CARD-3 or CARD-4 gene) into which a deletion, addition or substitution has been introduced to thereby alter, e.g., functionally disrupt, the CARD-3 or CARD-4 gene. In an 25 embodiment, the vector is designed such that, upon homologous recombination, the endogenous CARD-3 or CARD-4 gene is functionally disrupted (i.e., no longer encodes a functional protein; also referred to as a "knock out" vector). Alternatively, the vector can be designed such 30 that, upon homologous recombination, the endogenous CARD-3 or CARD-4 gene is mutated or otherwise altered but still encodes functional protein (e.g., the upstream regulatory region can be altered to thereby alter the expression of the endogenous CARD-3 or CARD-4 protein). 35 In the homologous recombination vector, the altered

- 70 -

portion of the CARD-3 or CARD-4 gene is flanked at its 5' and 3' ends by additional nucleic acid of the CARD-3 or CARD-4 gene to allow for homologous recombination to occur between the exogenous CARD-3 or CARD-4 gene carried by the vector and an endogenous CARD-3 or CARD-4 gene in an embryonic stem cell. The additional flanking CARD-3 or CARD-4 nucleic acid is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see, e.g., Thomas and Capecchi (1987) Cell 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced CARD-3 or CARD-4 gene has homologously recombined with the endogenous CARD-3 or CARD-4 gene are selected (see, e.g., Li et al. (1992) Cell 69:915). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see, e.g., Bradley in Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination vectors and homologous recombinant animals are described further in Bradley (1991) Current Opinion in Bio/Technology 2:823-829 and in PCT Publication Nos. WO 90/11354, WO 91/01140, WO 92/0968, and WO 93/04169.

In another embodiment, transgenic non-humans animals can be produced which contain selected systems

- 71 -

which allow for regulated expression of the transgene. One example of such a system is the cre/loxP recombinase system of bacteriophage P1. For a description of the cre/loxP recombinase system, see, e.g., Lakso et al.

- 5 (1992) Proc. Natl. Acad. Sci. USA 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman et al. (1991) Science 251:1351-1355. If a cre/loxP recombinase system is used to regulate expression of the transgene,
- 10 animals containing transgenes encoding both the Cre recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a
- 15 selected protein and the other containing a transgene encoding a recombinase.

- Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut et al. (1997) Nature
- 20 385:810-813 and PCT Publication Nos. WO 97/07668 and WO 97/07669. In brief, a cell, e.g., a somatic cell, from the transgenic animal can be isolated and induced to exit the growth cycle and enter Go phase. The quiescent cell can then be fused, e.g., through the use of electrical
- 25 pulses, to an enucleated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyte and then transferred to pseudopregnant female foster animal. The offspring borne
- 30 of this female foster animal will be a clone of the animal from which the cell, e.g., the somatic cell, is isolated.

- 72 -

#### IV. Pharmaceutical Compositions

The CARD-3 or CARD-4 nucleic acid molecules, CARD-3 or CARD-4 proteins, and anti-CARD-3 or CARD-4 antibodies (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as

- 73 -

acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral  
5 preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the  
10 extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL? (BASF; Parsippany, NJ) or phosphate buffered saline (PBS). In all cases,  
15 the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier  
20 can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a  
25 coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens,  
30 chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable

- 74 -

compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by  
5 incorporating the active compound (e.g., a CARD-3 or  
CARD-4 protein or anti-CARD-3 or CARD-4 antibody) in the  
required amount in an appropriate solvent with one or a  
combination of ingredients enumerated above, as required,  
followed by filtered sterilization. Generally,  
10 dispersions are prepared by incorporating the active  
compound into a sterile vehicle which contains a basic  
dispersion medium and the required other ingredients from  
those enumerated above. In the case of sterile powders  
for the preparation of sterile injectable solutions, the  
15 preferred methods of preparation are vacuum drying and  
freeze-drying which yields a powder of the active  
ingredient plus any additional desired ingredient from a  
previously sterile-filtered solution thereof.

Oral compositions generally include an inert  
20 diluent or an edible carrier. They can be enclosed in  
gelatin capsules or compressed into tablets. For the  
purpose of oral therapeutic administration, the active  
compound can be incorporated with excipients and used in  
the form of tablets, troches, or capsules. Oral  
25 compositions can also be prepared using a fluid carrier  
for use as a mouthwash, wherein the compound in the fluid  
carrier is applied orally and swished and expectorated or  
swallowed. Pharmaceutically compatible binding agents,  
and/or adjuvant materials can be included as part of the  
30 composition. The tablets, pills, capsules, troches and  
the like can contain any of the following ingredients, or  
compounds of a similar nature: a binder such as  
microcrystalline cellulose, gum tragacanth or gelatin; an  
excipient such as starch or lactose, a disintegrating  
35 agent such as alginic acid, Primogel, or corn starch; a

- 75 -

lubricant such as magnesium stearate or Sterotes; a  
glidant such as colloidal silicon dioxide; a sweetening  
agent such as sucrose or saccharin; or a flavoring agent  
such as peppermint, methyl salicylate, or orange  
5 flavoring. For administration by inhalation, the  
compounds are delivered in the form of an aerosol spray  
from pressured container or dispenser which contains a  
suitable propellant, e.g., a gas such as carbon dioxide,  
or a nebulizer.

10           Systemic administration can also be by  
transmucosal or transdermal means. For transmucosal or  
transdermal administration, penetrants appropriate to the  
barrier to be permeated are used in the formulation.  
Such penetrants are generally known in the art, and  
15 include, for example, for transmucosal administration,  
detergents, bile salts, and fusidic acid derivatives.  
Transmucosal administration can be accomplished through  
the use of nasal sprays or suppositories. For  
transdermal administration, the active compounds are  
20 formulated into ointments, salves, gels, or creams as  
generally known in the art.

The compounds can also be prepared in the form of  
suppositories (e.g., with conventional suppository bases  
such as cocoa butter and other glycerides) or retention  
25 enemas for rectal delivery.

In one embodiment, the active compounds are  
prepared with carriers that will protect the compound  
against rapid elimination from the body, such as a  
controlled release formulation, including implants and  
30 microencapsulated delivery systems. Biodegradable,  
biocompatible polymers can be used, such as ethylene  
vinyl acetate, polyanhydrides, polyglycolic acid,  
collagen, polyorthoesters, and polylactic acid. Methods  
for preparation of such formulations will be apparent to  
35 those skilled in the art. The materials can also be



- 76 -

obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as  
5 pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or  
10 parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active  
15 compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the  
20 particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors.  
25 Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (U.S. Patent 5,328,470) or by stereotactic injection (see, e.g., Chen et al. (1994) Proc. Natl. Acad. Sci. USA 91:3054-3057). The pharmaceutical preparation of the  
30 gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g.  
35 retroviral vectors, the pharmaceutical preparation can

- 77 -

include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with  
5 instructions for administration.

#### V. Uses and Methods of the Invention

The nucleic acid molecules, proteins, protein homologues, and antibodies described herein can be used in one or more of the following methods: a) screening  
10 assays; b) detection assays (e.g., chromosomal mapping, tissue typing, forensic biology), c) predictive medicine (e.g., diagnostic assays, prognostic assays, monitoring clinical trials, and pharmacogenomics); and d) methods of treatment (e.g., therapeutic and prophylactic). A CARD-3  
15 or CARD-4 protein interacts with other cellular proteins and can thus be used for (i) regulation of cellular proliferation; (ii) regulation of cellular differentiation; and (iii) regulation of cell survival. The isolated nucleic acid molecules of the invention can  
20 be used to express CARD-3 or CARD-4 protein (e.g., via a recombinant expression vector in a host cell in gene therapy applications), to detect CARD-3 or CARD-4 mRNA (e.g., in a biological sample) or a genetic lesion in a CARD-3 or CARD-4 gene, and to modulate CARD-3 or CARD-4  
25 activity. In addition, the CARD-3 or CARD-4 proteins can be used to screen drugs or compounds which modulate the CARD-3 or CARD-4 activity or expression as well as to treat disorders characterized by insufficient or excessive production of CARD-3 or CARD-4 protein or  
30 production of CARD-3 or CARD-4 protein forms which have decreased or aberrant activity compared to CARD-3 or CARD-4 wild type protein. In addition, the anti-CARD-3 or CARD-4 antibodies of the invention can be used to

- 78 -

detect and isolate CARD-3 or CARD-4 proteins and modulate CARD-3 or CARD-4 activity.

This invention further pertains to novel agents identified by the above-described screening assays and  
5 uses thereof for treatments as described herein.

#### A. Screening Assays

The invention provides a method (also referred to herein as a "screening assay") for identifying modulators, i.e., candidate or test compounds or agents  
10 (e.g., peptides, peptidomimetics, small molecules or other drugs) which bind to CARD-3 or CARD-4 proteins or biologically active portions thereof or have a stimulatory or inhibitory effect on, for example, CARD-3 or CARD-4 expression or CARD-3 or CARD-4 activity. An  
15 example of a biologically active portion of human CARD-4 is amino acids 1-145 encoding the CARD domain which is sufficient to exhibit CARD-3-binding activity as described in Example 7. Amino acids 406-953 of human CARD4L comprising the LRR domain represent a biologically  
20 active portion of CARD-4L because they possess hNUDC-binding activity as described in Example 8.

In one embodiment, the invention provides assays for screening candidate or test compounds which bind to or modulate the activity of a CARD-3 or CARD-4 proteins  
25 or polypeptides or biologically active portions thereof. The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable  
30 parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide

- 79 -

libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam (1997) Anticancer Drug Des. 12:145). Examples of methods for the synthesis of  
5 molecular libraries can be found in the art, for example in: DeWitt et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90:6909; Erb et al. (1994) Proc. Natl. Acad. Sci. USA 91:11422; Zuckermann et al. (1994). J. Med. Chem. 37:2678; Cho et al. (1993) Science 261:1303; Carrell et  
10 al. (1994) Angew. Chem. Int. Ed. Engl. 33:2059; Carell et al. (1994) Angew. Chem. Int. Ed. Engl. 33:2061; and Gallop et al. (1994) J. Med. Chem. 37:1233.

Libraries of compounds may be presented in solution (e.g., Houghten (1992) Bio/Techniques  
15 13:412-421), or on beads (Lam (1991) Nature 354:82-84), chips (Fodor (1993) Nature 364:555-556), bacteria (U.S. Patent No. 5,223,409), spores (Patent Nos. 5,571,698; 5,403,484; and 5,223,409), plasmids (Cull et al. (1992) Proc. Natl. Acad. Sci. USA 89:1865-1869) or on phage  
20 (Scott and Smith (1990) Science 249:386-390; Devlin (1990) Science 249:404-406; Cwirla et al. (1990) Proc. Natl. Acad. Sci. 87:6378-6382; and Felici (1991) J. Mol. Biol. 222:301-310).

Determining the ability of the test compound to  
25 modulate the activity of CARD-3 or CARD-4 or a biologically active portion thereof can be accomplished, for example, by determining the ability of the CARD-3 or CARD-4 protein to bind to or interact with a CARD-3 or  
CARD-4 target molecule. As used herein, a "target  
30 molecule" is a molecule with which a CARD-3 or CARD-4 protein binds or interacts in nature, for example, a molecule associated with the internal surface of a cell membrane or a cytoplasmic molecule. A CARD-3 or CARD-4 target molecule can be a non-CARD-3 or CARD-4 molecule or  
35 a CARD-3 or CARD-4 protein or polypeptide of the present

- 80 -

invention. In one embodiment, a CARD-3 or CARD-4 target molecule is a component of an apoptotic signal transduction pathway, e.g., CARD-3 and CARD-4. The target, for example, can be a second intracellular  
5 protein which has catalytic activity or a protein which facilitates the association of downstream signaling molecules with CARD-3 or CARD-4. In another embodiment, CARD-3 or CARD-4 target molecules include CARD-3 because CARD-3 was found to bind to CARD-4 (Examples 7 and 12)  
10 and hNUDC because hNUDC was found to bind to CARD-4 (Example 8).

Determining the ability of the test compound to modulate the activity of CARD-3 or CARD-4 or a biologically active portion thereof can be accomplished,  
15 for example, by determining the ability of the CARD-3 or CARD-4 protein to bind to or interact with any of the specific proteins listed in the previous paragraph as CARD-3 or CARD-4 target molecules. In another embodiment, CARD-3 or CARD-4 target molecules include all  
20 proteins that bind to a CARD-3 or CARD-4 protein or fragment thereof in a two-hybrid system binding assay which can be used without undue experimentation to isolate such proteins from cDNA or genomic two-hybrid system libraries. For example, Example 7 describes the  
25 use of the CARD-4 CARD domain region to identify CARD-3 in a two-hybrid screen and Example 8 describes the use of the CARD-4 LRR region to identify hNUDC in a two-hybrid screen. The binding assays described in this section could be cell-based or cell free (described  
30 subsequently).

Determining the ability of the CARD-3 or CARD-4 protein to bind to or interact with a CARD-3 or CARD-4 target molecule can be accomplished by one of the methods described above for determining direct binding. In an  
35 embodiment, determining the ability of the CARD-3 or

- 81 -

CARD-4 protein to bind to or interact with a CARD-3 or CARD-4 target molecule can be accomplished by determining the activity of the target molecule. For example, the activity of the target molecule can be determined by

5 detecting induction of a cellular second messenger of the target (e.g., intracellular Ca<sup>2+</sup>, diacylglycerol, IP<sub>3</sub>, etc.), detecting catalytic/enzymatic activity of the target on an appropriate substrate, detecting the induction of a reporter gene (e.g., a CARD-3 or

10 CARD-4-responsive regulatory element operatively linked to a nucleic acid encoding a detectable marker, e.g. luciferase), or detecting a cellular response, for example, cell survival, cellular differentiation, or cell proliferation. For example, in Example 12 CARD-4 is

15 shown to bind to CARD-3 and in Example 10, by monitoring a cellular response, CARD-4 is shown to enhance caspase 9 activity, cell death or apoptosis. Because CARD-3 and CARD-4 enhance caspase 9 activity, CARD-3 or CARD-4 activity can be monitored by assaying the caspase

20 9-mediated apoptosis cellular response or caspase 9 enzymatic activity. In addition, and in another embodiment, genes induced by CARD-3 or CARD-4 expression could be identified by expressing CARD-3 or CARD-4 in a cell line and conducting a transcriptional profiling

25 experiment wherein the mRNA expression patterns of the cell line transformed with an empty expression vector and the cell line transformed with a CARD-3 or CARD-4 expression vector are compared. The promoters of genes induced by CARD-3 or CARD-4 expression could be

30 operatively linked to reporter genes suitable for screening such as luciferase, secreted alkaline phosphatase, or beta-galactosidase and the resulting constructs could be introduced into appropriate expression vectors. A recombinant cell line containing

35 CARD-3 or CARD-4 and transfected with an expression

- 82 -

vector containing a CARD-3 or CARD-4 responsive promoter, operatively linked to a reporter gene could be used to identify test compounds that modulate CARD-3 or CARD-4 activity by assaying the expression of the reporter gene in response to contacting the recombinant cell line with test compounds. CARD-3 or CARD-4 agonists can be identified as increasing the expression of the reporter gene and CARD-3 or CARD-4 antagonists can be identified as decreasing the expression of the reporter gene.

10 In another embodiment of the invention, the ability of a test compound to modulate the activity of CARD-3, CARD-4, or biologically active portions thereof can be determined by assaying the ability of the test compound to modulate CARD-3 or CARD-4-dependent pathways or processes where the CARD-3 or CARD-4 target proteins that mediate the CARD-3 or CARD-4 effect are known or unknown. Potential CARD-3 or CARD-4-dependent pathways or processes include but are not limited to the modulation of cellular signal transduction pathways and their related second messenger molecules (e.g., intracellular  $\text{Ca}^{2+}$ , diacylglycerol, IP3, cAMP etc.), cellular enzymatic activities, cellular responses (e.g., cell survival, cellular differentiation, or cell proliferation), or the induction or repression of cellular or heterologous mRNAs or proteins. CARD-3 or CARD-4-dependent pathways or processes could be assayed by standard cell-based or cell free assays appropriate for the specific pathway or process under study. For example, Example 9 describes how expression of CARD-4S or CARD-4L in 293T cells induces the NF- $\kappa$ B pathway as determined by the measurement of a cotransfected NF- $\kappa$ B pathway luciferase reporter gene. In another embodiment, cells cotransfected with CARD-4 and the NF- $\kappa$ B luciferase reporter gene could be contacted with a test compound and test compounds that block CARD-4 activity could be

- 83 -

identified by their reduction of CARD-4-dependent NF- $\kappa$ B pathway luciferase reporter gene expression. Test compounds that agonize CARD-4 would be expected to increase reporter gene expression. In another embodiment, CARD-4 could be expressed in a cell line and the recombinant CARD-4-expressing cell line could be contacted with a test compound. Test compounds that inhibit CARD-4 activity could be indentified by their reduction of CARD-4-depended NF- $\kappa$ B pathway stimulation as measured by the assay of a NF- $\kappa$ B pathway reporter gene, NF- $\kappa$ B nuclear localization, I $\kappa$ B phosphorylation or proteolysis, or other standard assays for NF- $\kappa$ B pathway activation known to those skilled in the art.

In yet another embodiment, an assay of the present invention is a cell-free assay comprising contacting a CARD-3 or CARD-4 protein or biologically active portion thereof with a test compound and determining the ability of the test compound to bind to the CARD-3 or CARD-4 protein or biologically active portion thereof. Binding of the test compound to the CARD-3 or CARD-4 protein can be determined either directly or indirectly as described above. In one embodiment, a competitive binding assay includes contacting the CARD-3 or CARD-4 protein or biologically active portion thereof with a compound known to bind CARD-3 or CARD-4 to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a CARD-3 or CARD-4 protein, wherein determining the ability of the test compound to interact with a CARD-3 or CARD-4 protein comprises determining the ability of the test compound to preferentially bind to CARD-3 or CARD-4 or biologically active portion thereof as compared to the known binding compound.

In another embodiment, an assay is a cell-free assay comprising contacting CARD-3 or CARD-4 protein or



- 84 -

biologically active portion thereof with a test compound and determining the ability of the test compound to modulate (e.g., stimulate or inhibit) the activity of the CARD-3 or CARD-4 protein or biologically active portion thereof. Determining the ability of the test compound to modulate the activity of CARD-3 or CARD-4 can be accomplished, for example, by determining the ability of the CARD-3 or CARD-4 protein to bind to a CARD-3 or CARD-4 target molecule by one of the methods described above for determining direct binding. In an alternative embodiment, determining the ability of the test compound to modulate the activity of CARD-3 or CARD-4 can be accomplished by determining the ability of the CARD-3 or CARD-4 protein to further modulate a CARD-3 or CARD-4 target molecule. For example, the catalytic/enzymatic activity of the target molecule on an appropriate substrate can be determined as previously described.

In yet another embodiment, the cell-free assay comprises contacting the CARD-3 or CARD-4 protein or biologically active portion thereof with a known compound which binds CARD-3 or CARD-4 to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a CARD-3 or CARD-4 protein, wherein determining the ability of the test compound to interact with a CARD-3 or CARD-4 protein comprises determining the ability of the CARD-3 or CARD-4 protein to preferentially bind to or modulate the activity of a CARD-3 or CARD-4 target molecule. The cell-free assays of the present invention are amenable to use of both the soluble form or the membrane-associated form of CARD-3 or CARD-4. A membrane-associated form of CARD-3 or CARD-4 refers to CARD-3 or CARD-4 that interacts with a membrane-bound target molecule. In the case of cell-free assays comprising the membrane-associated form of CARD-3 or

- 85 -

CARD-4, it may be desirable to utilize a solubilizing agent such that the membrane-associated form of CARD-3 or CARD-4 is maintained in solution. Examples of such solubilizing agents include non-ionic detergents such as  
5 n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-114, Thesit®, Isotridecypoly(ethylene glycol ether)n,  
3-[(3-cholamidopropyl)dimethylamminio]-1-propane  
10 sulfonate (CHAPS),  
3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or  
N-dodecyl-N,N-dimethyl-3-ammonio-1-propane sulfonate.

In more than one embodiment of the above assay  
15 methods of the present invention, it may be desirable to immobilize either CARD-3 or CARD-4 or its target molecule to facilitate separation of complexed from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of a test  
20 compound to CARD-3 or CARD-4, or interaction of CARD-3 or CARD-4 with a target molecule in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples  
25 of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-transferase/ CARD-3 or CARD-4  
30 fusion proteins or glutathione-S-transferase/target fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical; St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the test compound or the test compound and either the non-adsorbed target protein or CARD-3 or  
35 CARD-4 protein, and the mixture incubated under

- 86 -

conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtitre plate wells are washed to remove any unbound components, the matrix

5 immobilized in the case of beads, complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of CARD-3 or CARD-4 binding or activity determined using standard techniques.

10 In an alternative embodiment, MYC or HA epitope tag CARD-3 or CARD-4 fusion proteins or MYC or HA epitope tag target fusion proteins can be adsorbed onto anti-MYC or anti-HA antibody coated microbeads or onto anti-MYC or anti-HA antibody coated microtitre plates, which are then

15 combined with the test compound or the test compound and either the non-adsorbed target protein or CARD-3 or CARD-4 protein, and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following

20 incubation, the beads or microtitre plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated

25 from the matrix, and the level of CARD-3 or CARD-4 binding or activity determined using standard techniques. Example 12 describes an HA epitope tagged CARD-4 protein that physically interacts in a coimmunoprecipitation assay with MYC epitope tagged CARD-3. In an embodiment

30 of the invention, HA epitope tagged CARD-4 could be used in combination with MYC epitope CARD-3 in the sort of protein-protein interaction assay described earlier in this paragraph.

Other techniques for immobilizing proteins on

35 matrices can also be used in the screening assays of the

- 87 -

invention. For example, either CARD-3 or CARD-4 or its target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated CARD-3 or CARD-4 or target molecules can be prepared from

5 biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals; Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with CARD-3 or CARD-4

10 or target molecules but which do not interfere with binding of the CARD-3 or CARD-4 protein to its target molecule can be derivatized to the wells of the plate, and unbound target or CARD-3 or CARD-4 trapped in the wells by antibody conjugation. Methods for detecting

15 such complexes, in addition to those described above for the GST-immobilized complexes and epitope tag immobilized complexes, include immunodetection of complexes using antibodies reactive with the CARD-3 or CARD-4 or target molecule, as well as enzyme-linked assays which rely on

20 detecting an enzymatic activity associated with the CARD-3 or CARD-4 or target molecule.

In another embodiment, modulators of CARD-3 or CARD-4 expression are identified in a method in which a cell is contacted with a candidate compound and the

25 expression of the CARD-3 or CARD-4 promoter, mRNA or protein in the cell is determined. The level of expression of CARD-3 or CARD-4 mRNA or protein in the presence of the candidate compound is compared to the level of expression of CARD-3 or CARD-4 mRNA or protein

30 in the absence of the candidate compound. The candidate compound can then be identified as a modulator of CARD-3 or CARD-4 expression based on this comparison. For example, when expression of CARD-3 or CARD-4 mRNA or protein is greater (statistically significantly greater)

35 in the presence of the candidate compound than in its

- 88 -

absence, the candidate compound is identified as a stimulator of CARD-3 or CARD-4 mRNA or protein expression. Alternatively, when expression of CARD-3 or CARD-4 mRNA or protein is less (statistically significantly less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of CARD-3 or CARD-4 mRNA or protein expression. The level of CARD-3 or CARD-4 mRNA or protein expression in the cells can be determined by methods described herein for detecting CARD-3 or CARD-4 mRNA or protein. The activity of the CARD-3 or CARD-4 promoter can be assayed by linking the CARD-3 or CARD-4 promoter to a reporter gene such as luciferase, secreted alkaline phosphatase, or beta-galactosidase and introducing the resulting construct into an appropriate vector, transfecting a host cell line, and measuring the activity of the reporter gene in response to test compounds. For example, two CARD-4-specific mRNAs were detected in a Northern blotting experiment, one of 4.6 kilobases and the other of 6.5-7.0 kilobases (Example 11). In Example 11, CARD-4-specific mRNA species were found to be widely distributed in the tissues and cell lines studied.

In yet another aspect of the invention, the CARD-3 or CARD-4 proteins can be used as "bait proteins" in a two-hybrid assay or three hybrid assay (see, e.g., U.S. Patent No. 5,283,317; Zervos et al. (1993) Cell 72:223-232; Madura et al. (1993) J. Biol. Chem. 268:12046-12054; Bartel et al. (1993) Bio/Techniques 14:920-924; Iwabuchi et al. (1993) Oncogene 8:1693-1696; and PCT Publication No. WO 94/10300), to identify other proteins, which bind to or interact with CARD-3 or CARD-4 ("CARD-3 or CARD-4-binding proteins" or "CARD-3 or CARD-4-bp") and modulate CARD-3 or CARD-4 activity. Such CARD-3 or CARD-4-binding proteins are also likely to be

- 89 -

involved in the propagation of signals by the CARD-3 or CARD-4 proteins as, for example, upstream or downstream elements of the CARD-3 or CARD-4 pathway. For example, Example 7 describes the construction of a two-hybrid screening bait construct including human CARD-4L amino acids 1-145 comprising the CARD domain and the use of this bait construct to screen human mammary gland and prostate gland two-hybrid libraries resulting in the identification of human CARD-3 as a CARD-4 interacting protein. In another example, Example 8 describes the construction of a two-hybrid screening bait construct including human CARD-4 amino acids 406-953 comprising the LRR domain and the use of this bait construct to screen a human mammary gland two-hybrid libraries resulting in the identification of hNUDC as a CARD-4 interacting protein.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, the gene that codes for CARD-3 or CARD-4 is fused to a gene encoding the DNA binding domain of a known transcription factor (e.g., GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact, in vivo, forming an CARD-3 or CARD-4-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (e.g., LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the

- 90 -

functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with CARD-3 or CARD-4.

In an embodiment of the invention, the ability of a test compound to modulate the activity of CARD-3, CARD-4, or a biologically active portion thereof can be determined by assaying the ability of the test compound to block the binding of CARD-3 and CARD-4 to their target proteins in a two-hybrid system assay. Example 7 describes a two-hybrid system assay for the interaction between CARD-3 and CARD-4 and Example 8 describes a two-hybrid system assay for the interaction between CARD-4 and its target protein hNUDC. To screen for test compounds that block the interaction between CARD-3 and CARD-4 and their target proteins, which include but are not limited to CARD-3, CARD-4, and hNUDC, a yeast two-hybrid screening strain coexpressing the interacting bait and prey constructs, for example, a CARD-4 bait construct and a CARD-3 prey construct as described in Example 7, is contacted with the test compound and the activity of the two-hybrid system reporter gene, usually HIS3, lacZ, or URA3 is assayed. If the strain remains viable but exhibits a significant decrease in reporter gene activity, this would indicate that the test compound has inhibited the interaction between the bait and prey proteins. This assay could be automated for high throughput drug screening purposes. In another embodiment of the invention, CARD-3 or CARD-4 and their target proteins could be configured in the reverse two-hybrid system (Vidal et al. (1996) Proc. Natl. Acad. Sci. USA 93:10321-6 and Vidal et al. (1996) Proc. Natl. Acad. Sci. USA 93:10315-20) designed specifically for efficient drug screening. In the reverse two-hybrid system, inhibition of a CARD-3 or CARD-4 physical interaction with a target protein would result in

- 91 -

induction of a reporter gene in contrast to the normal two-hybrid system where inhibition of CARD-3 or CARD-4 physical interaction with a target protein would lead to reporter gene repression. The reverse two-hybrid system  
5 is preferred for drug screening because reporter gene induction is more easily assayed than reporter gene repression.

Alternative embodiments of the invention are proteins found to physically interact with proteins that  
10 bind to CARD-3 or CARD-4. CARD-3 or CARD-4 interactors, including but not limited to hNUDC and CARD-3, could be configured into two-hybrid system baits and used in two-hybrid screens to identify additional members of the CARD-3 and CARD-4 pathway. The interactors of CARD-3 or  
15 CARD-4 interactors identified in this way could be useful targets for therapeutic intervention in CARD-4 related diseases and pathologies and an assay of their enzymatic or binding activity could be useful for the identification of test compounds that modulate CARD-3 or  
20 CARD-4 activity.

This invention further pertains to novel agents identified by the above-described screening assays and uses thereof for treatments as described herein.

#### B. Detection Assays

25 Portions or fragments of the cDNA sequences identified herein (and the corresponding complete gene sequences) can be used in numerous ways as polynucleotide reagents. For example, these sequences can be used to:  
(i) map their respective genes on a chromosome; and,  
30 thus, locate gene regions associated with genetic disease; (ii) identify an individual from a minute biological sample (tissue typing); and (iii) aid in forensic identification of a biological sample. These applications are described in the subsections below.



- 92 -

### 1. Chromosome Mapping

Once the sequence (or a portion of the sequence) of a gene has been isolated, this sequence can be used to map the location of the gene on a chromosome.

5 Accordingly, CARD-3 or CARD-4 nucleic acid molecules described herein or fragments thereof, can be used to map the location of CARD-3 or CARD-4 genes on a chromosome. The mapping of the CARD-3 or CARD-4 sequences to  
10 chromosomes is an important first step in correlating these sequences with genes associated with disease.

Briefly, CARD-3 or CARD-4 genes can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp in length) from the CARD-3 or CARD-4 sequences. Computer  
15 analysis of CARD-3 or CARD-4 sequences can be used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers can then be used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids  
20 containing the human gene corresponding to the CARD-3 or CARD-4 sequences will yield an amplified fragment. For example, in Example 6, human CARD-4-specific PCR primers were used to screen DNAs from a somatic cell hybrid panel showing that human CARD-4 maps to chromosome 7 close to  
25 the SHGC-31928 genetic marker.

Somatic cell hybrids are prepared by fusing somatic cells from different mammals (e.g., human and mouse cells). As hybrids of human and mouse cells grow and divide, they gradually lose human chromosomes in  
30 random order, but retain the mouse chromosomes. By using media in which mouse cells cannot grow, because they lack a particular enzyme, but human cells can, the one human chromosome that contains the gene encoding the needed enzyme, will be retained. By using various media, panels  
35 of hybrid cell lines can be established. Each cell line

- 93 -

in a panel contains either a single human chromosome or a small number of human chromosomes, and a full set of mouse chromosomes, allowing easy mapping of individual genes to specific human chromosomes. (D'Eustachio et al. 5 (1983) Science 220:919-924). Somatic cell hybrids containing only fragments of human chromosomes can also be produced by using human chromosomes with translocations and deletions.

PCR mapping of somatic cell hybrids is a rapid 10 procedure for assigning a particular sequence to a particular chromosome. Three or more sequences can be assigned per day using a single thermal cycler. Using the CARD-3 or CARD-4 sequences to design oligonucleotide primers, sublocalization can be achieved with panels of 15 fragments from specific chromosomes. Other mapping strategies which can similarly be used to map a CARD-3 or CARD-4 sequence to its chromosome include in situ hybridization (described in Fan et al. (1990) Proc. Natl. Acad. Sci. USA 87:6223-27), pre-screening with labeled 20 flow-sorted chromosomes, and pre-selection by hybridization to chromosome specific cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a DNA sequence to a metaphase chromosomal spread can further be used to provide a precise chromosomal location in one 25 step. Chromosome spreads can be made using cells whose division has been blocked in metaphase by a chemical like colcemid that disrupts the mitotic spindle. The chromosomes can be treated briefly with trypsin, and then stained with Giemsa. A pattern of light and dark bands 30 develops on each chromosome, so that the chromosomes can be identified individually. The FISH technique can be used with a DNA sequence as short as 500 or 600 bases. However, clones larger than 1,000 bases have a higher likelihood of binding to a unique chromosomal location 35 with sufficient signal intensity for simple detection.

- 94 -

Preferably 1,000 bases, and more preferably 2,000 bases will suffice to get good results at a reasonable amount of time. For a review of this technique, see Verma et al., (Human Chromosomes: A Manual of Basic Techniques  
5 (Pergamon Press, New York, 1988)).

Reagents for chromosome mapping can be used individually to mark a single chromosome or a single site on that chromosome, or panels of reagents can be used for marking multiple sites and/or multiple chromosomes.

10 Reagents corresponding to noncoding regions of the genes actually are preferred for mapping purposes. Coding sequences are more likely to be conserved within gene families, thus increasing the chance of cross hybridizations during chromosomal mapping.

15 Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man, available on-line  
20 through Johns Hopkins University Welch Medical Library). The relationship between genes and disease, mapped to the same chromosomal region, can then be identified through linkage analysis (co-inheritance of physically adjacent genes), described in, e.g., Egeland et al. (1987) Nature,  
25 325:783-787.

Moreover, differences in the DNA sequences between individuals affected and unaffected with a disease associated with the CARD-3 or CARD-4 gene can be determined. If a mutation is observed in some or all of  
30 the affected individuals but not in any unaffected individuals, then the mutation is likely to be the causative agent of the particular disease. Comparison of affected and unaffected individuals generally involves first looking for structural alterations in the  
35 chromosomes such as deletions or translocations that are

- 95 -

visible from chromosome spreads or detectable using PCR based on that DNA sequence. Ultimately, complete sequencing of genes from several individuals can be performed to confirm the presence of a mutation and to distinguish mutations from polymorphisms.

## 2. Tissue Typing

The CARD-3 or CARD-4 sequences of the present invention can also be used to identify individuals from minute biological samples. The United States military, for example, is considering the use of restriction fragment length polymorphism (RFLP) for identification of its personnel. In this technique, an individual's genomic DNA is digested with one or more restriction enzymes, and probed on a Southern blot to yield unique bands for identification. This method does not suffer from the current limitations of "Dog Tags" which can be lost, switched, or stolen, making positive identification difficult. The sequences of the present invention are useful as additional DNA markers for RFLP (described in U.S. Patent 5,272,057).

Furthermore, the sequences of the present invention can be used to provide an alternative technique which determines the actual base-by-base DNA sequence of selected portions of an individual's genome. Thus, the CARD-3 or CARD-4 sequences described herein can be used to prepare two PCR primers from the 5' and 3' ends of the sequences. These primers can then be used to amplify an individual's DNA and subsequently sequence it.

Panels of corresponding DNA sequences from individuals, prepared in this manner, can provide unique individual identifications, as each individual will have a unique set of such DNA sequences due to allelic differences. The sequences of the present invention can be used to obtain such identification sequences from individuals and from tissue. The CARD-3 or CARD-4

- 96 -

sequences of the invention uniquely represent portions of the human genome. Allelic variation occurs to some degree in the coding regions of these sequences, and to a greater degree in the noncoding regions. It is estimated  
5 that allelic variation between individual humans occurs with a frequency of about once per each 500 bases. Each of the sequences described herein can, to some degree, be used as a standard against which DNA from an individual can be compared for identification purposes. Because  
10 greater numbers of polymorphisms occur in the noncoding regions, fewer sequences are necessary to differentiate individuals. The noncoding sequences of SEQ ID NO:1, SEQ ID NO:7, SEQ ID NO:25 and SEQ ID NO:42 can comfortably provide positive individual identification with a panel  
15 of perhaps 10 to 1,000 primers which each yield a noncoding amplified sequence of 100 bases. If predicted coding sequences, such as those in SEQ ID NO:3, SEQ ID NO:9, and SEQ ID NO:27 are used, a more appropriate number of primers for positive individual identification  
20 would be 500-2,000.

If a panel of reagents from CARD-3 or CARD-4 sequences described herein is used to generate a unique identification database for an individual, those same reagents can later be used to identify tissue from that  
25 individual. Using the unique identification database, positive identification of the individual, living or dead, can be made from extremely small tissue samples.

### 3. Use of Partial CARD-3 or CARD-4 Sequences in Forensic Biology

30 DNA-based identification techniques can also be used in forensic biology. Forensic biology is a scientific field employing genetic typing of biological evidence found at a crime scene as a means for positively identifying, for example, a perpetrator of a crime. To  
35 make such an identification, PCR technology can be used

- 97 -

to amplify DNA sequences taken from very small biological samples such as tissues, e.g., hair or skin, or body fluids, e.g., blood, saliva, or semen found at a crime scene. The amplified sequence can then be compared to a  
5 standard, thereby allowing identification of the origin of the biological sample.

The sequences of the present invention can be used to provide polynucleotide reagents, e.g., PCR primers, targeted to specific loci in the human genome, which can  
10 enhance the reliability of DNA-based forensic identifications by, for example, providing another "identification marker" (i.e. another DNA sequence that is unique to a particular individual). As mentioned above, actual base sequence information can be used for  
15 identification as an accurate alternative to patterns formed by restriction enzyme generated fragments. Sequences targeted to noncoding regions of SEQ ID NO:1, SEQ ID NO:7, and SEQ ID NO:25 are particularly appropriate for this use as greater numbers of  
20 polymorphisms occur in the noncoding regions, making it easier to differentiate individuals using this technique. Examples of polynucleotide reagents include the CARD-3 or CARD-4 sequences or portions thereof, e.g., fragments derived from the noncoding regions of SEQ ID NO:1, SEQ ID  
25 NO:7, or SEQ ID NO:25 which have a length of at least 20 or 30 bases.

The CARD-3 or CARD-4 sequences described herein can further be used to provide polynucleotide reagents, e.g., labeled or labelable probes which can be used in,  
30 for example, an in situ hybridization technique, to identify a specific tissue, e.g., brain tissue. This can be very useful in cases where a forensic pathologist is presented with a tissue of unknown origin. Panels of such CARD-3 or CARD-4 probes can be used to identify  
35 tissue by species and/or by organ type.

- 98 -

In a similar fashion, these reagents, e.g., CARD-3 or CARD-4 primers or probes can be used to screen tissue culture for contamination (i.e., screen for the presence of a mixture of different types of cells in a culture).

5           **C. Predictive Medicine**

The present invention also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, pharmacogenomics, and monitoring clinical trials are used for prognostic (predictive) purposes to thereby treat an individual prophylactically. Accordingly, one aspect of the present invention relates to diagnostic assays for determining CARD-3 or CARD-4 protein and/or nucleic acid expression as well as CARD-3 or CARD-4 activity, in the context of a biological sample (e.g., blood, serum, cells, tissue) to thereby determine whether an individual is afflicted with a disease or disorder, or is at risk of developing a disorder, associated with aberrant CARD-3 or CARD-4 expression or activity. The invention also provides for prognostic (or predictive) assays for determining whether an individual is at risk of developing a disorder associated with CARD-3 or CARD-4 protein, nucleic acid expression or activity. For example, mutations in a CARD-3 or CARD-4 gene can be assayed in a biological sample. Such assays can be used for prognostic or predictive purpose to thereby prophylactically treat an individual prior to the onset of a disorder characterized by or associated with CARD-3 or CARD-4 protein, nucleic acid expression or activity.

30           Another aspect of the invention provides methods for determining CARD-3 or CARD-4 protein, nucleic acid expression or CARD-3 or CARD-4 activity in an individual to thereby select appropriate therapeutic or prophylactic agents for that individual (referred to herein as

"pharmacogenomics"). Pharmacogenomics allows for the selection of agents (e.g., drugs) for therapeutic or prophylactic treatment of an individual based on the genotype of the individual (e.g., the genotype of the individual examined to determine the ability of the individual to respond to a particular agent.)

Yet another aspect of the invention pertains to monitoring the influence of agents (e.g., drugs or other compounds) on the expression or activity of CARD-3 or CARD-4 in clinical trials.

These and other agents are described in further detail in the following sections.

## 1. Diagnostic Assays

An exemplary method for detecting the presence or absence of CARD-3 or CARD-4 in a biological sample involves obtaining a biological sample from a test subject and contacting the biological sample with a compound or an agent capable of detecting CARD-3 or CARD-4 protein or nucleic acid (e.g., mRNA, genomic DNA) that encodes CARD-3 or CARD-4 protein such that the presence of CARD-3 or CARD-4 is detected in the biological sample. An agent for detecting CARD-3 or CARD-4 mRNA or genomic DNA is a labeled nucleic acid probe capable of hybridizing to CARD-3 or CARD-4 mRNA or genomic DNA. The nucleic acid probe can be, for example, a full-length CARD-3 or CARD-4 nucleic acid, such as the nucleic acid of SEQ ID NO: 1 or 3, SEQ ID NO: 7 or 9, SEQ ID NO:25 or 27, or a portion thereof, such as an oligonucleotide of at least 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to CARD-3 or CARD-4 mRNA or genomic DNA, or a human CARD-4 splice variant such as the nucleic acid of SEQ ID NO:38 or SEQ ID NO:40. Other suitable probes for use in the diagnostic assays of the invention are described herein. For example, Example



- 100 -

11 describes the use of a nucleic acid probe to detect CARD-4 mRNAs in human tissues and cell lines and the probe used in this experiment could be used for a diagnostic assay.

5           An agent for detecting CARD-3 or CARD-4 protein can be an antibody capable of binding to CARD-3 or CARD-4 protein, preferably an antibody with a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. For example, polypeptides corresponding to  
10 amino acids 128-139 and 287-298 of human CARD-4L were used to immunize rabbits and produce polyclonal antibodies that specifically recognize human CARD-4L. An intact antibody, or a fragment thereof (e.g., Fab or F(ab')<sub>2</sub>) can be used. The term "labeled", with regard to  
15 the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (i.e., physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is  
20 directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin. The term "biological  
25 sample" is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. That is, the detection method of the invention can be used to detect CARD-3 or CARD-4 mRNA, protein, or genomic DNA in  
30 a biological sample in vitro as well as in vivo. For example, in vitro techniques for detection of CARD-3 or CARD-4 mRNA include Northern hybridizations and in situ hybridizations. For example, Example 11 contains the use of a human CARD-4L nucleic acid probe for a Northern  
35 blotting analysis of mRNA species encoded by human

- 101 -

CARD-4L detected in RNA samples from human tissues and cell lines. *In vitro* techniques for detection of CARD-3 or CARD-4 protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence. *In vitro* techniques for detection of CARD-3 or CARD-4 genomic DNA include Southern hybridizations. Furthermore, *in vivo* techniques for detection of CARD-3 or CARD-4 protein include introducing into a subject a labeled anti-CARD-3 or CARD-4 antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

In one embodiment, the biological sample contains protein molecules from the test subject. Alternatively, the biological sample can contain mRNA molecules from the test subject or genomic DNA molecules from the test subject. An biological sample is a peripheral blood leukocyte sample isolated by conventional means from a subject.

In another embodiment, the methods further involve obtaining a control biological sample from a control subject, contacting the control sample with a compound or agent capable of detecting CARD-3 or CARD-4 protein, mRNA, or genomic DNA, such that the presence of CARD-3 or CARD-4 protein, mRNA or genomic DNA is detected in the biological sample, and comparing the presence of CARD-3 or CARD-4 protein, mRNA or genomic DNA in the control sample with the presence of CARD-3 or CARD-4 protein, mRNA or genomic DNA in the test sample.

The invention also encompasses kits for detecting the presence of CARD-3 or CARD-4 in a biological sample (a test sample). Such kits can be used to determine if a subject is suffering from or is at increased risk of developing a disorder associated with aberrant expression of CARD-3 or CARD-4 (e.g., an immunological disorder).

- 102 -

For example, the kit can comprise a labeled compound or agent capable of detecting CARD-3 or CARD-4 protein or mRNA in a biological sample and means for determining the amount of CARD-3 or CARD-4 in the sample (e.g., an  
5 anti-CARD-3 or CARD-4 antibody or an oligonucleotide probe which binds to DNA encoding CARD-3 or CARD-4, e.g., SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:25 or SEQ ID NO:27). Kits may also include instruction for observing that the tested subject is  
10 suffering from or is at risk of developing a disorder associated with aberrant expression of CARD-3 or CARD-4 if the amount of CARD-3 or CARD-4 protein or mRNA is above or below a normal level.

For antibody-based kits, the kit may comprise, for  
15 example: (1) a first antibody (e.g., attached to a solid support) which binds to CARD-3 or CARD-4 protein; and, optionally, (2) a second, different antibody which binds to CARD-3 or CARD-4 protein or the first antibody and is conjugated to a detectable agent.

20 For oligonucleotide-based kits, the kit may comprise, for example: (1) a oligonucleotide, e.g., a detectably labelled oligonucleotide, which hybridizes to a CARD-3 or CARD-4 nucleic acid sequence or (2) a pair of primers useful for amplifying a CARD-3 or CARD-4 nucleic  
25 acid molecule.

The kit may also comprise, e.g., a buffering agent, a preservative, or a protein stabilizing agent. The kit may also comprise components necessary for detecting the detectable agent (e.g., an enzyme or a  
30 substrate). The kit may also contain a control sample or a series of control samples which can be assayed and compared to the test sample contained. Each component of the kit is usually enclosed within an individual container and all of the various containers are within a  
35 single package along with instructions for observing

- 103 -

whether the tested subject is suffering from or is at risk of developing a disorder associated with aberrant expression of CARD-3 or CARD-4.

## 2. Prognostic Assays

5       The methods described herein can furthermore be utilized as diagnostic or prognostic assays to identify subjects having or at risk of developing a disease or disorder associated with aberrant CARD-3 or CARD-4 expression or activity. For example, the assays  
10 described herein, such as the preceding diagnostic assays or the following assays, can be utilized to identify a subject having or at risk of developing a disorder associated with CARD-3 or CARD-4 protein, nucleic acid expression or activity. Alternatively, the prognostic  
15 assays can be utilized to identify a subject having or at risk for developing such a disease or disorder. Thus, the present invention provides a method in which a test sample is obtained from a subject and CARD-3 or CARD-4 protein or nucleic acid (e.g., mRNA, genomic DNA) is  
20 detected, wherein the presence of CARD-3 or CARD-4 protein or nucleic acid is diagnostic for a subject having or at risk of developing a disease or disorder associated with aberrant CARD-3 or CARD-4 expression or activity. As used herein, a "test sample" refers to a  
25 biological sample obtained from a subject of interest. For example, a test sample can be a biological fluid (e.g., serum), cell sample, or tissue. Furthermore, the prognostic assays described herein can be used to determine whether a subject can be administered an agent  
30 (e.g., an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate) to treat a disease or disorder associated with aberrant CARD-3 or CARD-4 expression or activity. For example, such methods can be used to determine whether a  
35 subject can be effectively treated with a specific agent

- 104 -

or class of agents (e.g., agents of a type which decrease CARD-3 or CARD-4 activity). Thus, the present invention provides methods for determining whether a subject can be effectively treated with an agent for a disorder  
5 associated with aberrant CARD-3 or CARD-4 expression or activity in which a test sample is obtained and CARD-3 or CARD-4 protein or nucleic acid is detected (e.g., wherein the presence of CARD-3 or CARD-4 protein or nucleic acid is diagnostic for a subject that can be administered the  
10 agent to treat a disorder associated with aberrant CARD-3 or CARD-4 expression or activity).

The methods of the invention can also be used to detect genetic lesions or mutations in a CARD-3 or CARD-4 gene, thereby determining if a subject with the lesioned  
15 gene is at risk for a disorder characterized by aberrant cell proliferation and/or differentiation. In preferred embodiments, the methods include detecting, in a sample of cells from the subject, the presence or absence of a genetic lesion characterized by at least one of an  
20 alteration affecting the integrity of a gene encoding a CARD-3 or CARD-4-protein, or the mis-expression of the CARD-3 or CARD-4 gene. For example, such genetic lesions can be detected by ascertaining the existence of at least one of 1) a deletion of one or more nucleotides from a  
25 CARD-3 or CARD-4 gene; 2) an addition of one or more nucleotides to a CARD-3 or CARD-4 gene; 3) a substitution of one or more nucleotides of a CARD-3 or CARD-4 gene, 4) a chromosomal rearrangement of a CARD-3 or CARD-4 gene; 5) an alteration in the level of a messenger RNA  
30 transcript of a CARD-3 or CARD-4 gene, 6) aberrant modification of a CARD-3 or CARD-4 gene, such as of the methylation pattern of the genomic DNA, 7) the presence of a non-wild type splicing pattern of a messenger RNA transcript of a CARD-3 or CARD-4 gene (e.g, caused by a  
35 mutation in a splice donor or splice acceptor site), 8) a

- 105 -

non-wild type level of a CARD-3 or CARD-4-protein, 9)  
allelic loss of a CARD-3 or CARD-4 gene, and 10)  
inappropriate post-translational modification of a CARD-3  
or CARD-4-protein. As described herein, there are a  
5 large number of assay techniques known in the art which  
can be used for detecting lesions in a CARD-3 or CARD-4  
gene. A biological sample is a peripheral blood  
leukocyte sample isolated by conventional means from a  
subject.

10 In certain embodiments, detection of the lesion  
involves the use of a probe/primer in a polymerase chain  
reaction (PCR) (see, e.g., U.S. Patent Nos. 4,683,195 and  
4,683,202), such as anchor PCR or RACE PCR, or,  
alternatively, in a ligation chain reaction (LCR) (see,  
15 e.g., Landegran et al. (1988) Science 241:1077-1080; and  
Nakazawa et al. (1994) Proc. Natl. Acad. Sci. USA  
91:360-364), the latter of which can be particularly  
useful for detecting point mutations in the CARD-3 or  
CARD-4-gene (see, e.g., Abravaya et al. (1995) Nucleic  
20 Acids Res. 23:675-682). This method can include the  
steps of collecting a sample of cells from a patient,  
isolating nucleic acid (e.g., genomic, mRNA or both) from  
the cells of the sample, contacting the nucleic acid  
sample with one or more primers which specifically  
25 hybridize to a CARD-3 or CARD-4 gene under conditions  
such that hybridization and amplification of the CARD-3  
or CARD-4-gene (if present) occurs, and detecting the  
presence or absence of an amplification product, or  
detecting the size of the amplification product and  
30 comparing the length to a control sample. It is  
anticipated that PCR and/or LCR may be desirable to use  
as a preliminary amplification step in conjunction with  
any of the techniques used for detecting mutations  
described herein.

- 106 -

Alternative amplification methods include: self sustained sequence replication (Guatelli et al. (1990) Proc. Natl. Acad. Sci. USA 87:1874-1878), transcriptional amplification system (Kwoh, et al. (1989) Proc. Natl. Acad. Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi et al. (1988) Bio/Technology 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In an alternative embodiment, mutations in a CARD-3 or CARD-4 gene from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence specific ribozymes (see, e.g., U.S. Patent No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

In other embodiments, genetic mutations in CARD-3 or CARD-4 can be identified by hybridizing a sample and control nucleic acids, e.g., DNA or RNA, to high density arrays containing hundreds or thousands of oligonucleotides probes (Cronin et al. (1996) Human Mutation 7:244-255; Kozal et al. (1996) Nature Medicine 2:753-759). For example, genetic mutations in CARD-3 or CARD-4 can be identified in two-dimensional arrays containing light-generated DNA probes as described in Cronin et al. supra. Briefly, a first hybridization

- 107 -

array of probes can be used to scan through long stretches of DNA in a sample and control to identify base changes between the sequences by making linear arrays of sequential overlapping probes. This step allows the  
5 identification of point mutations. This step is followed by a second hybridization array that allows the characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of  
10 parallel probe sets, one complementary to the wild-type gene and the other complementary to the mutant gene.

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the CARD-3 or CARD-4 gene and detect  
15 mutations by comparing the sequence of the sample CARD-3 or CARD-4 with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on techniques developed by Maxam and Gilbert ((1977) Proc. Natl. Acad. Sci. USA 74:560) or Sanger  
20 ((1977) Proc. Natl. Acad. Sci. USA 74:5463). It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays ((1995) Bio/Techniques 19:448), including sequencing by mass spectrometry (see, e.g., PCT  
25 Publication No. WO 94/16101; Cohen et al. (1996) Adv. Chromatogr. 36:127-162; and Griffin et al. (1993) Appl. Biochem. Biotechnol. 38:147-159).

Other methods for detecting mutations in the CARD-3 or CARD-4 gene include methods in which protection  
30 from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers et al. (1985) Science 230:1242). In general, the art technique of "mismatch cleavage" starts by providing heteroduplexes of formed by hybridizing (labeled) RNA or DNA containing the  
35 wild-type CARD-3 or CARD-4 sequence with potentially



- 108 -

mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to basepair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with S1 nuclease to enzymatically digesting the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels to determine the site of mutation. See, e.g., Cotton et al (1988) Proc. Natl Acad Sci USA 85:4397; Saleeba et al (1992) Methods Enzymol. 217:286-295. In an embodiment, the control DNA or RNA can be labeled for detection.

In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes) in defined systems for detecting and mapping point mutations in CARD-3 or CARD-4 cDNAs obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu et al. (1994) Carcinogenesis 15:1657-1662). According to an exemplary embodiment, a probe based on a CARD-3 or CARD-4 sequence, e.g., a wild-type CARD-3 or CARD-4 sequence, is hybridized to a cDNA or other DNA product from a test cell(s). The duplex is treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like. See, e.g., U.S. Patent No. 5,459,039.

- 109 -

In other embodiments, alterations in electrophoretic mobility will be used to identify mutations in CARD-3 or CARD-4 genes. For example, single strand conformation polymorphism (SSCP) may be used to  
5 detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita et al. (1989) Proc Natl. Acad. Sci USA: 86:2766, see also Cotton (1993) Mutat. Res. 285:125-144; and Hayashi (1992) Genet Anal Tech Appl 9:73-79). Single-stranded DNA fragments of  
10 sample and control CARD-3 or CARD-4 nucleic acids will be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a  
15 single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In an embodiment, the subject method  
20 utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen et al. (1991) Trends Genet 7:5).

In yet another embodiment, the movement of mutant  
25 or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers et al. (1985) Nature 313:495). When DGGE is used as the method of analysis, DNA will be modified to insure that it does not  
30 completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and  
35 Reissner (1987) Biophys Chem 265:12753).

- 110 -

Examples of other techniques for detecting point mutations include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example,

5 oligonucleotide primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki et al. (1986) Nature 324:163); Saiki et al. (1989) Proc. Natl Acad. Sci  
10 USA 86:6230). Such allele specific oligonucleotides are hybridized to PCR amplified target DNA or a number of different mutations when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA.

15 Alternatively, allele specific amplification technology which depends on selective PCR amplification may be used in conjunction with the instant invention. Oligonucleotides used as primers for specific amplification may carry the mutation of interest in the  
20 center of the molecule (so that amplification depends on differential hybridization) (Gibbs et al. (1989) Nucleic Acids Res. 17:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner (1993)  
25 Tibtech 11:238). In addition, it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection (Gasparini et al. (1992) Mol. Cell Probes 6:1). It is anticipated that in certain embodiments amplification may also be  
30 performed using Taq ligase for amplification (Barany (1991) Proc. Natl. Acad. Sci USA 88:189). In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect the presence of a known mutation at a specific

- 111 -

site by looking for the presence or absence of amplification.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe nucleic acid or antibody reagent described herein, which may be conveniently used, e.g., in clinical settings to diagnose patients exhibiting symptoms or family history of a disease or illness involving a CARD-3 or CARD-4 gene.

Furthermore, any cell type or tissue, preferably peripheral blood leukocytes, in which CARD-3 or CARD-4 is expressed may be utilized in the prognostic assays described herein.

### 3. Pharmacogenomics

Agents, or modulators which have a stimulatory or inhibitory effect on CARD-3 or CARD-4 activity (e.g., CARD-3 or CARD-4 gene expression) as identified by a screening assay described herein can be administered to individuals to treat (prophylactically or therapeutically) disorders (e.g., an immunological disorder) associated with aberrant CARD-3 or CARD-4 activity. In conjunction with such treatment, the pharmacogenomics (i.e., the study of the relationship between an individual's genotype and that individual's response to a foreign compound or drug) of the individual may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, the pharmacogenomics of the individual permits the selection of effective agents (e.g., drugs) for prophylactic or therapeutic treatments based on a consideration of the individual's genotype. Such pharmacogenomics can further be used to determine appropriate dosages and therapeutic regimens.

- 112 -

Accordingly, the activity of CARD-3 or CARD-4 protein, expression of CARD-3 or CARD-4 nucleic acid, or mutation content of CARD-3 or CARD-4 genes in an individual can be determined to thereby select appropriate agent(s) for  
5 therapeutic or prophylactic treatment of the individual.

Pharmacogenomics deals with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See, e.g., Linder (1997) Clin. Chem.  
10 43(2):254-266. In general, two types of pharmacogenetic conditions can be differentiated. Genetic conditions transmitted as a single factor altering the way drugs act on the body (altered drug action) or genetic conditions transmitted as single factors altering the way the body  
15 acts on drugs (altered drug metabolism). These pharmacogenetic conditions can occur either as rare defects or as polymorphisms. For example, glucose-6-phosphate dehydrogenase deficiency (G6PD) is a common inherited enzymopathy in which the main clinical  
20 complication is haemolysis after ingestion of oxidant drugs (anti-malarials, sulfonamides, analgesics, nitrofurans) and consumption of fava beans.

As an illustrative embodiment, the activity of drug metabolizing enzymes is a major determinant of both  
25 the intensity and duration of drug action. The discovery of genetic polymorphisms of drug metabolizing enzymes (e.g., N-acetyltransferase 2 (NAT 2) and cytochrome P450 enzymes CYP2D6 and CYP2C19) has provided an explanation as to why some patients do not obtain the expected drug  
30 effects or show exaggerated drug response and serious toxicity after taking the standard and safe dose of a drug. These polymorphisms are expressed in two phenotypes in the population, the extensive metabolizer (EM) and poor metabolizer (PM). The prevalence of PM is  
35 different among different populations. For example, the

- 113 -

gene coding for CYP2D6 is highly polymorphic and several mutations have been identified in PM, which all lead to the absence of functional CYP2D6. Poor metabolizers of CYP2D6 and CYP2C19 quite frequently experience  
5 exaggerated drug response and side effects when they receive standard doses. If a metabolite is the active therapeutic moiety, PM show no therapeutic response, as demonstrated for the analgesic effect of codeine mediated by its CYP2D6-formed metabolite morphine. The other  
10 extreme are the so called ultra-rapid metabolizers who do not respond to standard doses. Recently, the molecular basis of ultra-rapid metabolism has been identified to be due to CYP2D6 gene amplification.

Thus, the activity of CARD-3 or CARD-4 protein,  
15 expression of CARD-3 or CARD-4 nucleic acid, ~~or mutation~~ content of CARD-3 or CARD-4 genes in an individual can be determined to thereby select appropriate agent(s) for therapeutic or prophylactic treatment of the individual. In addition, pharmacogenetic studies can be used to apply  
20 genotyping of polymorphic alleles encoding drug-metabolizing enzymes to the identification of an individual's drug responsiveness phenotype. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus  
25 enhance therapeutic or prophylactic efficiency when treating a subject with a CARD-3 or CARD-4 modulator, such as a modulator identified by one of the exemplary screening assays described herein.

#### 4. Monitoring of Effects During Clinical Trials

30 Monitoring the influence of agents (e.g., drugs, compounds) on the expression or activity of CARD-3 or CARD-4 (e.g., the ability to modulate aberrant cell proliferation and/or differentiation) can be applied not only in basic drug screening, but also in clinical  
35 trials. For example, the effectiveness of an agent

subjects exhibit CARD-4 activity. In such cases, expression, protein level, or activity of CARD-3 or CARD-4 and 15 genes that have been implicated in, for example, cellular proliferation disorder can be used as "out" or markers of the immune responsiveness of a particular cell.

For example, and not by way of limitation, genes, 20 including CARD-3 or CARD-4, that are modulated in cells by treatment with an agent (e.g., compound, drug or small molecule) which modulates CARD-3 or CARD-4 activity (e.g., identified in a screening assay as described herein) can be identified. Thus, to study the effect of 25 agents on cellular proliferation disorders, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of CARD-3 or CARD-4 and other genes implicated in the 30 expression pattern) can be quantified by Northern blot analysis or RT-PCR, as described herein, or alternatively by measuring the amount of protein produced, by the methods as described herein, or by measuring levels of activity of CARD-3 or CARD-4 or other 35 In this way, the gene expression pattern can be

ects expression, protein activity of CARD-3 or CARD-4 activity. In studies that have been implicated in, genes that have been implicated in, cellular proliferation disorder can be used out" or markers of the immune responsiveness of particular cell.

For example, and not by way of limitation, genes, by treatment with an agent (e.g., compound, drug or small molecule) which modulates CARD-3 or CARD-4, that are modulated in cells (e.g., identified in a screening assay as described herein) can be identified. Thus, to study the effect of 25 agents on cellular proliferation disorders, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of CARD-3 or CARD-4 and other genes implicated in the disorder. The levels of gene expression (i.e., a gene expression pattern) can be quantified by Northern blot analysis or RT-PCR, as described herein, or alternatively by measuring the amount of protein produced, by one of the methods as described herein, or by measuring the levels of activity of CARD-3 or CARD-4 or other genes. 35 In this way, the gene expression pattern can serve as a

- 115 -

marker, indicative of the physiological response of the cells to the agent. Accordingly, this response state may be determined before, and at various points during, treatment of the individual with the agent.

5           In an embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (e.g., an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate identified by the  
10 screening assays described herein) comprising the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level of expression of a CARD-3 or CARD-4 protein, mRNA, or genomic DNA in the preadministration  
15 sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the CARD-3 or CARD-4 protein, mRNA, or genomic DNA in the post-administration samples; (v) comparing the level of expression or activity of the  
20 CARD-3 or CARD-4 protein, mRNA, or genomic DNA in the pre-administration sample with the CARD-3 or CARD-4 protein, mRNA, or genomic DNA in the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example,  
25 increased administration of the agent may be desirable to increase the expression or activity of CARD-3 or CARD-4 to higher levels than detected, i.e., to increase the effectiveness of the agent. Alternatively, decreased administration of the agent may be desirable to decrease  
30 expression or activity of CARD-3 or CARD-4 to lower levels than detected, i.e., to decrease the effectiveness of the agent.



- 116 -

### C. Methods of Treatment

The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or  
5 having a disorder associated with aberrant CARD-3 or CARD-4 expression or activity.

#### 1. Prophylactic Methods

In one aspect, the invention provides a method for preventing in a subject, a disease or condition  
10 associated with an aberrant CARD-3 or CARD-4 expression or activity, by administering to the subject an agent which modulates CARD-3 or CARD-4 expression or at least one CARD-3 or CARD-4 activity. Subjects at risk for a disease which is caused or contributed to by aberrant  
15 CARD-3 or CARD-4 expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the CARD-3 or CARD-4  
20 aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of CARD-3 or CARD-4 aberrancy, for example, a CARD-3 or CARD-4 agonist or CARD-3 or CARD-4 antagonist agent can be used for treating the subject. The  
25 appropriate agent can be determined based on screening assays described herein. Activities of CARD-3 or CARD-4 that could be modulated for prophylactic purposes include, but are not limited to, 1) CARD-3 or CARD-4 gene or protein expression, for example, see Example 11 for a  
30 description of the mRNA expression pattern of human CARD-4; 2) CARD-3 or CARD-4 binding to a target protein, for example, see Examples 7, 8, and 12 for a description of proteins known to bind to CARD-3 or CARD-4; 3) CARD-4 regulation of NF- $\kappa$ B as described in Example 9; and 4)

- 117 -

CARD-3 and CARD-4 enhancement of caspase 9 activity as described in Example 10.

## 2. Therapeutic Methods

Another aspect of the invention pertains to  
5 methods of modulating CARD-3 or CARD-4 expression or activity for therapeutic purposes. The modulatory method of the invention involves contacting a cell with an agent that modulates one or more of the activities of CARD-3 or CARD-4 protein activity associated with the cell. An  
10 agent that modulates CARD-3 or CARD-4 protein activity can be an agent as described herein, such as a nucleic acid or a protein, a naturally-occurring cognate ligand of a CARD-3 or CARD-4 protein, a peptide, a CARD-3 or CARD-4 peptidomimetic, or other small molecule. In one  
15 embodiment, the agent stimulates one or more of the biological activities of CARD-3 or CARD-4 protein. Examples of such stimulatory agents include active CARD-3 or CARD-4 protein and a nucleic acid molecule encoding CARD-3 or CARD-4 that has been introduced into the cell.  
20 In another embodiment, the agent inhibits one or more of the biological activities of CARD-3 or CARD-4 protein. Examples of such inhibitory agents include antisense CARD-3 or CARD-4 nucleic acid molecules and anti-CARD-3 or CARD-4 antibodies. These modulatory methods can be  
25 performed in vitro (e.g., by culturing the cell with the agent) or, alternatively, in vivo (e.g., by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder characterized by aberrant  
30 expression or activity of a CARD-3 or CARD-4 protein or nucleic acid molecule. In one embodiment, the method involves administering an agent (e.g., an agent identified by a screening assay described herein), or combination of agents that modulates (e.g., upregulates  
35 or downregulates) CARD-3 or CARD-4 expression or

- 118 -

activity. In another embodiment, the method involves administering a CARD-3 or CARD-4 protein or nucleic acid molecule as therapy to compensate for reduced or aberrant CARD-3 or CARD-4 expression or activity. Activities of  
5 CARD-3 or CARD-4 that could be modulated for therapeutic purposes include, but are not limited to, 1) CARD-3 or CARD-4 gene or protein expression, for example, see Example 11 for a description of the mRNA expression pattern of human CARD-4; 2) CARD-3 or CARD-4 binding to a  
10 target protein, for example, see Examples 7, 8, and 12 for a description of proteins known to bind to CARD-3 or CARD-4; 3) CARD-4 regulation of NF- $\kappa$ B as described in Example 9; and 4) CARD-4 enhancement of caspase 9 activity as described in Example 10.

15 Stimulation of CARD-3 or CARD-4 activity is desirable in situations in which CARD-3 or CARD-4 is abnormally downregulated and/or in which increased CARD-3 or CARD-4 activity is likely to have a beneficial effect. Conversely, inhibition of CARD-3 or CARD-4 activity is  
20 desirable in situations in which CARD-3 or CARD-4 is abnormally upregulated, e.g., in myocardial infarction, and/or in which decreased CARD-3 or CARD-4 activity is likely to have a beneficial effect. Since CARD-4 may play be involved in the processing of cytokines,  
25 inhibiting the activity or expression CARD4- may be beneficial in patients that have aberrant inflammation.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and  
30 published patent applications cited throughout this application are hereby incorporated by reference.

- 119 -

EXAMPLES**Example 1: Isolation and Characterization of full-length Human CARD-3 and CARD-4L/S cDNAs.**

A profile of known CARD domains was used to search  
5 databases of cDNA sequences and partial cDNA sequences  
using TBLASTN (Washington University; version 2.0,  
BLOSUM62 search matrix). This search led to the  
identification of CARD-3. Using CARD-3 to search  
databases of cDNA sequences and partial cDNA sequences,  
10 another potential CARD cDNA was found. This cDNA  
sequence was used to screen a human umbilical vein  
endothelial library (HUVE) and a clone containing the  
partial CARD-4S was identified. The human umbilical vein  
endothelial library was then rescreened using a probe  
15 designed against the partial CARD-4S sequence and a clone  
containing the CARD-4L sequence was identified.

**Example 2: Characterization of CARD-3 AND CARD-4L/S Proteins.**

In this example, the predicted amino acid  
20 sequences of human CARD-3 and CARD-4L/S proteins were  
compared to amino acid sequences of known proteins and  
various motifs were identified. For example, the CARD  
domains of CARD-3 and CARD-4 were aligned (Figure 7) with  
the CARD domains of ARC-CARD (SEQ ID NO:31), cIAP1-CARD  
25 (SEQ ID NO:32) and cIAP2-CARD (SEQ ID NO:33). In  
addition, the molecular weight of the human CARD-3 and  
CARD-4L/S proteins were predicted.

The human CARD-3 cDNA was isolated as described  
above (Figure 1; SEQ ID NO:1) and encodes a 540 amino  
30 acid protein (Figure 2: SEQ ID NO:2). CARD-3 also  
includes one predicted kinase domain (amino acid 1 to  
amino acid 300 of SEQ ID NO:2; SEQ ID NO:4), which is  
followed by a predicted linker domain (amino acid 301 to  
amino acid 431 of SEQ ID NO:2; SEQ ID NO:5) and a

- 120 -

predicted CARD domain (amino acid 432 to amino acid 540 of SEQ ID NO:2; SEQ ID NO:6).

The human CARD-4L cDNA was isolated as described above (Figure 3; SEQ ID NO:7) and has a 2859 nucleotide  
5 open reading frame (nucleotides 245-3103 of SEQ ID NO:7; SEQ ID NO:9) which encodes a 953 amino acid protein (Figure 4; SEQ ID NO:8). CARD-4L protein has a predicted CARD domain (amino acids 15-114; SEQ ID NO:10). CARD-4L is also predicted to have a nucleotide binding domain  
10 which extends from about amino acid 198 to about amino acid 397 of SEQ ID NO:8; SEQ ID NO:11, a predicted Walker Box "A", which extends from about amino acid 202 to about amino acid 209 of SEQ ID NO:8; SEQ ID NO:12, a predicted Walker Box "B", which extends from about amino acid 280  
15 to about amino acid 284, of SEQ ID NO:8; SEQ ID NO:13, a predicted kinase 1a (P-loop) domain, which extends from about amino acid 197 to about amino acid 212 of SEQ ID NO:8; SEQ ID NO:46, a predicted kinase 2 domain, which extends from about amino acid 273 to about amino acid 288  
20 of SEQ ID NO:8; SEQ ID NO:47, a predicted kinase 3a subdomain, which extends from about amino acid 327 to about amino acid 338 of SEQ ID NO:8; SEQ ID NO:14, ten predicted Leucine-rich repeats which extend from about amino acid 674 to about amino acid 950 of SEQ ID NO:8.  
25 The first Leucine-rich repeat is predicted to extend from about amino acid 674 to about amino acid 701 of SEQ ID NO:8; SEQ ID NO:15. The second Leucine-rich repeat is predicted to extend from about amino acid 702 to about amino acid 727 of SEQ ID NO:8; SEQ ID NO:16. The third  
30 Leucine-rich repeat is predicted to extend from about amino acid 728 to about amino acid 754 of SEQ ID NO:8; SEQ ID NO:17. The fourth Leucine-rich repeat is predicted to extend from about amino acid 755 to about amino acid 782 of SEQ ID NO:8; SEQ ID NO:18. The fifth  
35 Leucine-rich repeat is predicted to extend from about

- 121 -

amino acid 783 to about amino acid 810 of SEQ ID NO:8; SEQ ID NO:19. The sixth Leucine-rich repeat is predicted to extend from about amino acid 811 to about amino acid 838 of SEQ ID NO:8; SEQ ID NO:20. The seventh

5 Leucine-rich repeat is predicted to extend from about amino acid 839 to about amino acid 866 of SEQ ID NO:8; SEQ ID NO:21. The eighth Leucine-rich repeat is predicted to extend from about amino acid 867 to about amino acid 894 of SEQ ID NO:8; SEQ ID NO:22. The ninth

10 Leucine-rich repeat is predicted to extend from about amino acid 895 to about amino acid 922 of SEQ ID NO:8; SEQ ID NO:23 and the tenth leucine-rich repeat is predicted to extend from about amino acid 923 to about amino acid 950 of SEQ ID NO:8; SEQ ID NO:24.

15 The human partial CARD-4S cDNA isolated as described above (Figure 5; SEQ ID NO:25) encodes a 490 amino acid protein (Figure 6; SEQ ID NO:26). CARD-4S includes one predicted partial CARD domain (amino acids 1-74 of SEQ ID NO:26). CARD-4S is also predicted to have

20 a P-Loop which extends from about amino acid 163 to about amino acid 170 of SEQ ID NO:26; SEQ ID NO:29, and a predicted Walker Box "B" which extends from about amino acid 241 to about amino acid 245 of SEQ ID NO:26; SEQ ID NO:30.

25 A plot showing the predicted structural features of CARD-4L is presented in Figure 8. This figure shows the predicted alpha regions (Garnier-Robinson and Chou-Fasman), the predicted beta regions (Garnier-Robinson and Chou-Fasman), the predicted turn

30 regions (Garnier-Robinson and Chou-Fasman) and the predicted coil regions (Garnier-Robinson and Chou-Fasman). Also included in the figure is a hydrophilicity plot (Kyte-Doolittle), the predicted alpha and beta-amphatic regions (Eisenberg), the predicted

35 flexible regions (Karplus-Schulz), the predicted

- 122 -

antigenic index (Jameson-Wolf) and the predicted surface probability plot (Emini).

A plot showing the predicted structural features of CARD-4S is also presented in Figure 9. This figure  
5 shows the predicted alpha regions (Garnier-Robinson and Chou-Fasman), the predicted beta regions (Garnier-Robinson and Chou-Fasman), the predicted turn regions (Garnier-Robinson and Chou-Fasman) and the predicted coil regions (Garnier-Robinson and  
10 Chou-Fasman). Also included in the figure is a hydrophilicity plot (Kyte-Doolittle), the predicted alpha and beta-amphatic regions (Eisenberg), the predicted flexible regions (Karplus-Schulz), the predicted antigenic index (Jameson-Wolf) and the predicted surface  
15 probability plot (Emini).

The predicted MW of CARD-3 is approximately 61 kDa. The predicted MW of CARD-4L is approximately 108 kDa.

### Example 3: Preparation of CARD-3 and CARD-4 Proteins

20 Recombinant CARD-3 and CARD-4 can be produced in a variety of expression systems. For example, the CARD-3 and CARD-4 peptides can be expressed as a recombinant glutathione-S-transferase (GST) fusion protein in E. coli and the fusion protein can be isolated and characterized.  
25 Specifically, as described above, CARD-3 or CARD-4 can be fused to GST and the fusion protein can be expressed in E. coli strain PEB199. Expression of the GST-CARD-3 or GST-CARD-4 fusion protein in PEB199 can be induced with IPTG. The recombinant fusion protein can be purified  
30 from crude bacterial lysates of the induced PEB199 strain by affinity chromatography on glutathione beads.

- 123 -

**Example 4: Identification of splice variants of CARD4.**

The 5' untranslated sequence from CARD-4L was used to search databases of cDNA sequences and partial cDNA sequences using BLASTN (Washington University; version 2.0, BLOSUM62 search matrix) for additional CARD-4 cDNA clones. This search led to the identification of two cDNA clones, clone Z from a human lymph node library and the Y clone from a human brain cDNA library. Both clones were sequenced and found to represent probable splice variants of CARD-4 that encode truncated CARD-4 proteins, Y encoding a 249 amino acid protein and Z encoding a 164 amino acid protein. Fig. 10 shows the nucleotide (SEQ ID NO:38) and Fig. 11 the predicted amino acid (SEQ ID NO:39) sequences of human CARD-4Y; Fig. 12 shows the nucleotide (SEQ ID NO:40) and Fig. 13 the amino acid (SEQ ID NO:41) sequences of human CARD-4Z; and Fig. 14 shows an alignment of the CARD-4L, CARD-4Y, and CARD-4Z amino acid sequences generated by the Clustal program using a PAM250 residue weight table.

**Example 5: Identification of murine CARD-4.**

The CARD-4 polypeptide sequence was used to search databases of cDNA sequences and partial cDNA sequences using the TBLASTN program (version 1.4, BLOSUM62 search matrix, and a word length of 3) for murine CARD-4 cDNA clones. This search led to the identification of a partial murine CARD-4 clone designated murine CARD-4L. The rapid identification of cDNA ends procedure (RACE) was applied to the 5' end of the murine CARD-4L clone to elucidate the 5' end of the murine CARD-4L cDNA. Fig. 15 shows the murine CARD-4L nucleotide sequence (SEQ ID NO:42), Figure 16 shows the murine CARD-4L amino acid sequence (SEQ ID NO:43), and Fig. 17 shows an alignment of the murine CARD-4L and human CARD-4L amino acid



- 124 -

sequences generated by the Clustal program using a PAM250 residue weight table.

**Example 6: Identification of the chromosomal location of human CARD-4.**

5 To determine the chromosomal location of the human CARD-4 gene, the polymerase chain reaction carried out with human CARD-4-specific primers card4t, with the 5' to 3' sequence agaaggtctggtcggcaaa (SEQ ID NO:44), and card4k, with the 5' to 3' sequence aagccctgagtgggaagca  
10 (SEQ ID NO:45), was used to screen DNAs from a commercially available somatic cell hybrid panel. This analysis showed that human CARD-4 maps to chromosome 7 close to the SHGC-31928 genetic marker.

**Example 7: Identification of CARD-3 in a yeast two-hybrid screen for proteins that physically interact with the CARD domain of human CARD-4.**

DNA encoding amino acids 1-145 of human CARD-4 comprising the CARD domain was cloned into a yeast two-hybrid screening vector to create a CARD-4,1-145-GAL4  
20 DNA-binding domain fusion for two-hybrid screening. The CARD-4,1-145-GAL4 DNA-binding domain fusion was used to screen human mammary gland and human prostate two-hybrid libraries for gene products that could physically associate with CARD-4,1-145. Twelve library plasmids  
25 expressing CARD4,1-145 interacting proteins were found to contain the CARD-domain containing protein CARD-3 thus establishing a direct or indirect physical interaction between CARD-4 and CARD-3.

In addition, DNA encoding amino acids 435-540 of  
30 CARD-3 comprising the CARD domain of CARD-3 (SEQ ID NO:6) was cloned into a yeast two-hybrid GAL4 transcriptional activation domain fusion vector to create a CARD-3,435-540-GAL4 transcriptional activation domain fusion. To

- 125 -

test whether the CARD domain of CARD-3 binds CARD-4,1-145, the CARD-3,435-540-GAL4 transcriptional activation domain fusion expression vector and the CARD-4,1-145-GAL4 DNA-binding domain fusion vector were  
5 cotransformed into a two-hybrid screening *Saccharomyces cerevisiae* (yeast) strain. The resulting cotransformed yeast strain expressed the two reporter genes that indicate a physical interaction between the two hybrid proteins in the experiment, in this case, the CARD-3,435-  
10 540-GAL4 transcriptional activation domain fusion protein and the CARD-4,1-145-GAL4 DNA-binding domain fusion protein. This experiment established a physical interaction between the CARD domain of CARD-3 and the CARD domain of CARD-4.

15 **Example 8: Identification of hNUDC in a yeast two-hybrid screen for proteins that physically interact with the LRR domain of human CARD-4.**

DNA encoding amino acids 406-953 of human CARD-4L comprising the LRR domain was cloned into a yeast  
20 two-hybrid screening vector to create a CARD-4,406-953-GAL4 DNA-binding domain fusion for two-hybrid screening. The CARD-4,406-953-GAL4 DNA-binding domain fusion was used to screen a human mammary gland two-hybrid library for gene products that  
25 could physically associate with CARD-4,406-953. One library plasmid expressing a CARD4,406-953 interacting protein was found to contain the hNUDC protein, the human ortholog of the rat NUDC protein that has been implicated in nuclear movement (Morris et al., Curr. Biol. 8:603  
30 [1998], Morris et al., Exp. Cell Res. 238:23 [1998]), thus establishing a physical interaction between CARD-4 and hNUDC.

- 126 -

**Example 9: Discovery of regulation by CARD-4 of NF- $\kappa$ B.**

The first group of experiments described in this Example were carried out to determine if CARD-4 can activate the NF- $\kappa$ B pathway. CARD-4 regulation of the  
5 NF- $\kappa$ B pathway is of interest because the NF- $\kappa$ B pathway is involved in many diseases described in (New England Journal of Medicine 336:1066 [1997]) and (American Journal of Cardiology 76:18C [1995]) and other references known to those skilled in the art. Participation of  
10 CARD-4 in the NF- $\kappa$ B pathway would make CARD-4 an attractive target for drugs that modulate the NF- $\kappa$ B pathway for treatment of NF- $\kappa$ B pathway-dependent diseases, conditions, and biological processes.

The first group of experiments showed specific  
15 CARD-4-mediated NF- $\kappa$ B pathway induction.

The second group of experiments described in this Example were carried out to determine if CARD-3, the NIK serine/threonine protein kinase (Su et al., EMBO J. 16:1279 [1997]), or the signal transduction protein TRAF6  
20 (Cao et al., Nature 383:443 [1996]), proteins known to participate in the induction of NF- $\kappa$ B (McCarthy et al., J. Biol. Chem. 273:16968 [1998]), are involved in transducing the CARD-4-dependent NF- $\kappa$ B pathway induction signal. It was found that CARD-3, NIK, and TRAF6 are all  
25 involved in transducing the CARD-4-mediated NF- $\kappa$ B pathway induction signal.

In nine transfection experiments, 293T cells coexpressing an NF- $\kappa$ B reporter plasmid and either pCI, pCI-CARD-4L (expressing CARD-4L), pCI-CARD-4S (expressing  
30 CARD-4S), pCI-APAF1 (expressing Apaf-1), pCI-APAFS (expressing an Apaf-1 variant lacking WD repeats), pCI-CARD-4LnoCARD (expressing CARD-4L without a CARD domain), pCI-CARD4LnoLRR (expressing CARD-4L without a LRR), pCI-CARD4LCARDonly (expressing CARD-4L CARD domain  
35 only), or pCI-CARD4NBSONly (expressing CARD-4L nucleotide

- 127 -

binding sequence only) were created. 293T cells were plated in 6-well plates (35 mm wells) and transfected 2 days later (90% confluency) with 1  $\mu$ g of NF- $\kappa$ B luciferase reporter plasmid (pNF- $\kappa$ B-Luc, 5 Stratagene), 200 ng of pCMV  $\beta$ -gal, 600 ng of pCI vector and 200 ng of indicated expression plasmids using SuperFect transfection reagent (Qiagen). For dominant-negative experiments, 2 ng of CARD4 expressing plasmid and 800 ng of dominant-negative plasmid were used. Cells 10 were harvested 48 h after transfection and luciferase activity in 1000-fold diluted cell extracts was determined using the Luciferase Assay System (Promega). In addition,  $\beta$ -galactosidase activities were determined and used to normalize transfection efficiency.

15           Relative luciferase activity was determined at the end of the experiment to assess NF- $\kappa$ B pathway activation by the gene expressed by the pCI-based plasmid in each transfected cell line. The cell lines containing pCI, pCI-APAFS, pCI-APAF $\Delta$ L, pCI-CARD-4LnoCARD, and pCI- 20 CARD4NB $\Delta$ Only had similar baseline levels of luciferase expression but the cell lines containing pCI-CARD-4L, pCI-CARD4LnoLRR, and pCI-CARD4LCARD $\Delta$ Only had luciferase expression about nine fold elevated relative to baseline and the cell line containing pCI-CARD4S had luciferase 25 expression sixteen fold elevated relative to baseline. This result demonstrates induction by CARD-4S and CARD-4L of the NF- $\kappa$ B pathway. This CARD-4 mediated NF- $\kappa$ B pathway induction is dependent on the CARD-4 CARD domain because the pCI-CARD-4noCARD construct expressing CARD-4 lacking 30 its CARD domain did not induce the luciferase reporter gene and pCI-CARD4LCARD $\Delta$ Only expressing the CARD-4 CARD domain did induce the luciferase reporter gene. Also, the CARD-4 LRR domains are not required for NF- $\kappa$ B pathway activation because pCI-CARD4LnoLRR expressing a CARD-4 35 mutant protein lacking LRR domains is able to induce the

- 128 -

luciferase reporter gene. In addition, the CARD-4 NBS domain is not sufficient for NF- $\kappa$ B pathway activation because pCI-CARD4NBSonly expressing CARD-4 NBS domain is not able to induce the luciferase reporter gene. In  
5 addition, the induction of the NF- $\kappa$ B pathway by CARD-4 is specific, as neither Apaf-expressing construct in this experiment induced luciferase activation.

In five transfection experiments, 293T cells coexpressing an NF- $\kappa$ B reporter plasmid (NF- $\kappa$ B-luciferase, Stratagene) and pCI-CARD-4L and either, no vector,  
10 pCI-TRAF6-DN (expressing a dominant negative version of TRAF-6), pCI-NIK-DN (expressing a dominant negative version of NIK kinase), pCI-CARD3CARDonly (expressing the CARD domain of CARD-3, which acts as a dominant negative  
15 version of CARD-3), or pCI-Bcl-XL (expressing the anti-apoptotic protein Bcl-XL) were created. TRAF6-DN, NIK-DN, and CARD3-CARDonly are dominant negative alleles of the TRAF6, NIK, and CARD3 genes, respectively. After 48 hours, cells were lysed and the relative luciferase  
20 activity was determined (Promega Kit) to assess NF- $\kappa$ B pathway activation by the genes expressed by the one or two pCI-based plasmids in each transfected cell line. The cell lines containing pCI-CARD-4L only or pCI-CARD-4L and pCI-Bcl-XL had relative luciferase reporter gene  
25 expression of about 18 units. The cell lines containing pCI-CARD-4L and pCI-TRAF6-DN, pCI-CARD-4L and pCI-NIK-DN, or pCI-CARD-4L and pCI-CARD3CARDonly had relative luciferase reporter gene expression of about 4 units. Inhibition of CARD-4L-mediated NF- $\kappa$ B pathway induction by  
30 TRAF6-DN, NIK-DN, and CARD-3CARDonly is specific as Bcl-XL did not inhibit CARD-4L-mediated NF- $\kappa$ B pathway induction.

These results demonstrate that dominant negative alleles of TRAF6, NIK and CARD-3 expressed, respectively,  
35 from pCI-TRAF6-DN, pCI-NIK-DN, and pCI-CARD3CARDonly

- 129 -

block induction of the NF- $\kappa$ B reporter gene by CARD-4L expression (pCI-CARD-4L) and suggest that TRAF6, NIK, and CARD-3 act downstream of CARD-4L to transduce the CARD-4L NF- $\kappa$ B pathway induction stimulus.

5           In an additional experiment, coexpression of CARD-4 and the CARD domain of RICK revealed that the CARD domain of RICK functions as a dominant negative mutant suggesting that RICK is a downstream mediator of CARD-4 function.

10 **Example 10: Discovery of CARD-4 enhancement of caspase 9 activity.**

          In ten transfection experiments, 293T cells coexpressing a beta galactosidase-expressing plasmid (pCMV  $\beta$ -gal from Stratagene) as a marker for viable cells  
15 and either pCI, pCI-CARD-3, pCI-APAF, pCI-CARD-4L, pCI-CARD-4S, pCI-CARD4LnoLRR, pCI-CARD4NBSONly, pCI-CARD4LCARDonly, pCI-CARD-4LnoCARD or pCI-casp9 (expressing caspase-9) were created. Transfections included 400 ng of pCMV  $\beta$ -gal, 800 ng of expression  
20 plasmid, and Superfect transfection reagent from Qiagen and were carried out according to the manufacturer's directions. After 40-48 hours, cells were fixed and stained for beta-galactosidase expression and cell viability was determined by counting the number of beta  
25 galactosidase positive cells. Expression of pCI, pCI-CARD-3, pCI-APAF, pCI-CARD-4L, pCI-CARD-4S, pCI-CARD4LnoLRR, pCI-CARD4NBSONly, pCI-CARD4LCARDonly, and pCI-CARD-4LnoCARD did not result in loss of cell viability. As expected, expression of pCI-casp9 in 293T  
30 cells resulted in a loss of viability of about 75% of the cells in the experiment.

          It was next tested whether pCI, pCI-CARD-3, pCI-APAF, pCI-CARD-4L, pCI-CARD-4S, pCI-CARD4LnoLRR, pCI-CARD4NBSONly, pCI-CARD4LCARDonly, or pCI-CARD-4LnoCARD

- 130 -

would regulate caspase 9-mediated apoptosis. In nine transfection experiments, 293T cells coexpressing a beta galactosidase-expressing plasmid as a marker for viable cells, pCI-casp9, and either pCI, pCI-CARD-3, pCI-APAF, 5 pCI-CARD-4L, pCI-CARD-4S, pCI-CARD4LnoLRR, pCI-CARD4NBSonly, pCI-CARD4LCARDonly, and pCI-CARD-4LnoCARD were created. After 40-48 hours, cells were fixed and stained for beta-galactosidase expression and cell viability was determined by counting the number of beta 10 galactosidase positive cells. Expression of pCI, pCI-CARD-4LnoCARD, and pCI-CARD4NBSonly in the caspase 9-expressing 293T cells had no effect on the caspase 9-induced apoptosis. However, pCI-CARD-3, pCI-CARD-4L, pCI-CARD-4S, pCI-CARD4LnoLRR, pCI-CARD4LCARDonly and, as 15 expected, pCI-APAF enhanced the level of caspase 9-induced apoptosis to 20 or less beta galactosidase positive cells per experiment from about 100 beta galactosidase positive cells per experiment.

This experiment demonstrated that CARD-4 can 20 enhance caspase 9-mediated apoptosis because coexpression of CARD-4L or CARD-4S with caspase-9 dramatically increases caspase-9 mediated apoptosis. Furthermore, the CARD-4 CARD domain (SEQ ID NO:10) is necessary and sufficient for CARD-4-mediated enhancement of caspase-9- 25 potentiated apoptosis because CARD-4L lacking its CARD domain (pCI-CARD-4LnoCARD) does not enhance caspase-9-mediated apoptosis while the CARD-4 CARD domain expressed alone (pCI-CARD4LCARDonly) does induce caspase-9 mediated apoptosis. In addition, the LRR present in CARD-4 is not 30 required for CARD-4 enhancement of caspase-9-mediated apoptosis because expression of a CARD-4 protein lacking the LRR (pCI-CARD4LnoLRR) still enhances caspase-9-mediated apoptosis. The CARD-4 NBS is not sufficient for CARD-4 enhancement of caspase-9-mediated apoptosis 35 because expression of the CARD-4 NBS only (pCI-

- 131 -

CARD4NBSonly) does not enhance caspase-9 mediated apoptosis. This experiment also demonstrates that CARD-3 can enhance caspase-9-mediated apoptosis.

As detailed below in Example 12, CARD-4 does not appear to interact directly with caspase-9, suggesting that potentiation of caspase-9 activity by CARD-4 is mediated by activation of downstream pathways.

**Example 11: Identification and tissue distribution of mRNA species expressed by the human CARD-4 gene.**

Northern analysis of mRNAs extracted from adult human tissues revealed a 4.6 kilobase mRNA band that was expressed in most tissues examined. Highest expression was observed heart, spleen, placenta and lung. CARD-4 was also observed to be expressed in fetal brain, lung, liver and kidney. Cancer cell lines expressing the 4.6 kilobase CARD-4 mRNA include HeLa, K562, Molt4, SW480, A549 and melanoma. A larger 6.5 to 7.0 kilobase CARD-4 mRNA was expressed in heart, spleen, lung, fetal lung, fetal liver, and in the Molt4 and SW480 cell lines.

**Example 12: Physical association of CARD-4 with CARD-3.**

CARD-4-specific PCR primers with the 3' primer encoding the HA epitope tag were used to amplify the CARD-4L gene epitope tagged with HA and this PCR product was cloned into the mammalian expression vector pCI. CARD-3-specific PCR primers with the 5' primer encoding the MYC epitope tag were used to amplify the CARD-3 gene epitope tagged with MYC and this PCR product was cloned into the mammalian expression vector pCI. CARD-3-specific PCR primers with the 5' primer encoding the MYC epitope tag were used to amplify the CARD-3 gene lacking the CARD domain (SEQ ID NO:6) epitope tagged with MYC and this PCR product was cloned into the mammalian expression vector pCI. Caspase 9-specific PCR primers with the 3' primer



- 132 -

encoding the MYC epitope tag were used to amplify the caspase 9 gene epitope tagged with MYC and this PCR product was cloned into the mammalian expression vector pCI. In three transfection experiments, 293T cells  
5 coexpressing pCI-CARD-4LcHA and either pCI-CARD3nMYC, pCI-CARD3noCARDnMYC, or pCI-casp9cMYC were created. Cells from each transfected line were lysed and an immunoprecipitation procedure was carried out on each lysate with an anti-MYC epitope tag antibody to  
10 precipitate the CARD-4LcHA expressed by each cell line and any physically associated proteins. Immunoprecipitated proteins were separated by electrophoresis on denaturing polyacrylamide gels, transferred to nylon filters, and probed with an anti-HA  
15 epitope tag antibody in a Western blotting experiment to determine whether the MYC-tagged protein that was coexpressed with the CARD-4LcHA protein had coimmunoprecipitated with the CARD-4LcHA protein. In this experiment, CARD-3 was found to coimmunoprecipitate  
20 with CARD-4 while CARD-3 lacking its CARD domain and caspase-9 did not coimmunoprecipitate with CARD-4. This experiment demonstrates that CARD-4 and CARD-3 physically associate and that CARD-3 requires its CARD domain to associate with CARD-4. In addition, CARD-4 appears to  
25 not associate with caspase-9.

### **Example 13: CARD-4 Genomic Sequence**

Figure 18 is depicts the 32042 nucleotide genomic sequence of CARD-4. This sequence is based the CARD-4 cDNA sequence described above and a BAC sequence (DBEST  
30 Accession No. AC006027). The CARD-4 cDNA sequence described above was used to correct three errors in the BAC sequence, including one error resulting in a frameshift. The CARD-4 genomic sequence of Figure 18 includes the following introns and exons: exon 1:

- 133 -

nucleotides 364-685, encoding amino acids 1-67 (start codon at nucleotides 485-487); intron 1: nucleotides 686-2094; exon 2: nucleotides 2095-2269, encoding amino acids 67-126; intron 2: nucleotides 2270-4365; exon 3: 5 nucleotides 366-6190, encoding amino acids 126-734; intron 3: nucleotides 6191-9024; exon 4: nucleotides 9025-9108, encoding amino acids 734-762; intron 4: nucleotides 9109-10355; exon 5: nucleotides 10356-10439, encoding amino acids 762-790; intron 5: nucleotides 10 10440-11181; exon 6: nucleotides 1182-11265, encoding amino acids 790-818; intron 6: nucleotides 11266-19749; exon 7: nucleotides 19750-19833, encoding amino acids 818-846; intron 7: nucleotides 19834-21324; exon 8: nucleotides 21325-21408, encoding amino acids 846-874; 15 intron 8: nucleotides 21409-24226; exon 9: nucleotides 24227-24310, amino acids 874-903; intron 9: nucleotides 24311-27948; exon 10: nucleotides 27949-28032, amino acids 903-930; intron 10: nucleotides 28033-31695; exon 11: nucleotides 31696-32024, encoding amino acids 930-953 20 (stop codon at nucleotides 31766-31768).

The introns in the CARD-4 genomic sequence contain consensus splice donor and acceptor sites (Molecular Cell Biology, Darnell et al., eds., 1996). The CARD-4 genomic sequence is useful for genetic identification and mapping 25 and identifying mutations, e.g., mutations in splice donor or splice acceptor sites.

### Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine 30 experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

- 134 -

What is claimed is:

1. An isolated nucleic acid molecule selected from the group consisting of:

- a) a nucleic acid comprising the nucleotide  
5 sequence of SEQ ID NO:38 or a complement thereof;
- b) a nucleic acid comprising the nucleotide sequence of SEQ ID NO:40 or a complement thereof;
- c) a nucleic acid comprising the nucleotide sequence of SEQ ID NO:42, or a complement thereof;
- 10 d) a nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:38;
- e) a nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence of SEQ ID  
15 NO:40;
- f) a nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:42;
- g) a nucleic acid comprising the nucleotide  
20 sequence of SEQ ID NO:38 or a complement thereof;
- h) a nucleic acid consisting of the nucleotide sequence of SEQ ID NO:40 or a complement thereof;
- i) a nucleic acid consisting of the nucleotide sequence of SEQ ID NO:42, or a complement thereof;
- 25 j) a nucleic acid molecule which encodes a polypeptide consisting of the amino acid sequence of SEQ ID NO:38;
- k) a nucleic acid molecule which encodes a polypeptide consisting of the amino acid sequence of SEQ  
30 ID NO:40; and
- l) a nucleic acid molecule which encodes a polypeptide consisting of the amino acid sequence of SEQ ID NO:42.

- 135 -

2. A host cell which contains one of the nucleic acid molecules of claim 1.

3. An isolated polypeptide selected from the group consisting of:

- 5 a) a polypeptide comprising the amino acid sequence of SEQ ID NO:39;
- b) a polypeptide comprising the amino acid sequence of SEQ ID NO:41;
- c) a polypeptide comprising the amino acid  
10 sequence of SEQ ID NO:43;
- d) a polypeptide consisting of the amino acid sequence of SEQ ID NO:39;
- e) a polypeptide consisting of the amino acid sequence of SEQ ID NO:41;
- 15 f) a polypeptide consisting of the amino acid sequence of SEQ ID NO:43;
- g) a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO:39, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID  
20 NO:39;
- h) a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO:41, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:41;
- 25 i) a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO:43, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO:43;
- j) a naturally occurring allelic variant of a  
30 polypeptide consisting of the amino acid sequence of SEQ ID NO:39, wherein the polypeptide is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule consisting of SEQ ID NO:38 under stringent conditions;

- 136 -

k) a naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence of SEQ ID NO:41, wherein the polypeptide is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule  
5 consisting of SEQ ID NO:40 under stringent conditions; and

l) a naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence of SEQ ID NO:43, wherein the polypeptide is encoded by a nucleic  
10 acid molecule which hybridizes to a nucleic acid molecule consisting of SEQ ID NO:42 under stringent conditions.

4. An antibody which selectively binds to any one of the polypeptides of claim 3.

5. A method for producing a polypeptide selected  
15 from the group consisting of:

- a) a polypeptide comprising the amino acid sequence of SEQ ID NO:39;
- b) a polypeptide comprising the amino acid sequence of SEQ ID NO:41;
- 20 c) a polypeptide comprising the amino acid sequence of SEQ ID NO:43;
- d) a polypeptide consisting of the amino acid sequence of SEQ ID NO:39;
- e) a polypeptide consisting of the amino acid  
25 sequence of SEQ ID NO:41;
- f) a polypeptide consisting of the amino acid sequence of SEQ ID NO:43;
- g) a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO:39, wherein the fragment  
30 comprises at least 15 contiguous amino acids of SEQ ID NO:39;
- h) a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO:41, wherein the fragment

- 137 -

comprises at least 15 contiguous amino acids of SEQ ID NO:41;

i) a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO:43, wherein the fragment  
5 comprises at least 15 contiguous amino acids of SEQ ID NO:43;

j) a naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence of SEQ ID NO:39, wherein the polypeptide is encoded by a nucleic  
10 acid molecule which hybridizes to a nucleic acid molecule consisting of SEQ ID NO:38 under stringent conditions;

k) a naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence of SEQ ID NO:41, wherein the polypeptide is encoded by a nucleic  
15 acid molecule which hybridizes to a nucleic acid molecule consisting of SEQ ID NO:40 under stringent conditions;  
and

l) a naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence of SEQ  
20 ID NO:43, wherein the polypeptide is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule consisting of SEQ ID NO:42 under stringent conditions;

comprising the step of culturing the host cell of claim 2 under conditions in which the nucleic acid  
25 molecule is expressed.

6. A method for detecting the presence of a polypeptide of claim 2 in a sample, comprising:

a) contacting the sample with a compound which selectively binds to a polypeptide of claim 2; and  
30 b) determining whether the compound binds to the polypeptide of claim 2 in the sample.

- 138 -

7. A method for detecting the presence of a nucleic acid molecule of claim 1 in a sample, comprising the steps of:

a) contacting the sample with a nucleic acid probe  
5 or primer which selectively hybridizes to the nucleic acid molecule; and

b) determining whether the nucleic acid probe or primer binds to a nucleic acid molecule in the sample.

8. A method for identifying a compound which  
10 binds to a polypeptide of claim 2 comprising the steps of:

a) contacting a polypeptide, or a cell expressing a polypeptide of claim 2 with a test compound; and

15 b) determining whether the polypeptide binds to the test compound.

9. The method of claim 8, wherein the binding of the test compound to the polypeptide is detected by a method selected from the group consisting of:

20 a) detection of binding by direct detecting of test compound/polypeptide binding;

b) detection of binding using a competition binding assay; and

25 c) detection of binding using an assay for CARD-4L or CARD-4S mediated signal transduction.

10. A method for modulating the activity of a polypeptide of claim 2 comprising contacting a polypeptide or a cell expressing a polypeptide of claim 2 with a compound which binds to the polypeptide in a  
30 sufficient concentration to modulate the activity of the polypeptide.

- 139 -

11. A method for identifying a compound which modulates the activity of a polypeptide of claim 2, comprising:

- a) contacting a polypeptide of claim 2 with a test compound; and
- b) determining the effect of the test compound on the activity of the polypeptide to thereby identify a compound which modulates the activity of the polypeptide.

12. A method for identifying a compound that blocks the interaction between a CARD-4 protein comprising a CARD-4 domain and a CARD-4-interacting protein comprising the steps of:

- a) incubating said CARD-4 protein and said interactor in the presence and absence of a test agent;
- b) determining whether said test agent reduces the binding of said CARD-4 protein and said interactor; and
- c) identifying a compound that blocks the interaction of said CARD-4 protein with said interactor when said compound reduces the binding of said CARD-4 protein with said interactor;

wherein said interactor is selected from the group consisting of CARD-3 and hNUDC and wherein said CARD-4 domain comprises amino acids 1-145 of an amino acid sequence selected from the group consisting of SEQ ID NO:8 and SEQ ID NO:43.

13. The method of claim 12, wherein the CARD-4 protein comprising a CARD-4 domain is selected from the group consisting of:

- a) a polypeptide comprising the amino acid sequence of SEQ ID NO:8;
- b) a polypeptide comprising the amino acid sequence of SEQ ID NO:39;



- 140 -

c) a polypeptide comprising the amino acid sequence of SEQ ID NO:41; and

d) a polypeptide comprising the amino acid sequence of SEQ ID NO:43.

5           14. The method of claim 12, wherein the CARD-4 protein and interactor are expressed in a recombinant prokaryotic or eukaryotic cell line or wherein the CARD-4 protein and interactor are isolated proteins or present in cell-free protein extracts.

10           15. A method for identifying a compound that inhibits the induction of the NF- $\kappa$ B pathway by a CARD-4 protein comprising the steps of:

a) incubating a recombinant cell line containing a vector expressing CARD-4 in the presence and absence of  
15 a test agent;

b) determining whether said test agent inhibits the induction of the NF- $\kappa$ B pathway by CARD-4; and

c) identifying a compound that inhibits the induction of the NF- $\kappa$ B pathway by CARD-4.

20           16. The method of claim 15, wherein the CARD-4 protein comprising a CARD-4 domain is selected from the group consisting of:

a) a polypeptide comprising the amino acid sequence of SEQ ID NO:8;

25           b) a polypeptide comprising the amino acid sequence of SEQ ID NO:39;

c) a polypeptide comprising the amino acid sequence of SEQ ID NO:41; and

30           d) a polypeptide comprising the amino acid sequence of SEQ ID NO:43.

- 141 -

17. The method of claim 15 further comprising:

a) incubating a recombinant cell line expressing CARD-4 and also expressing an NF- $\kappa$ B pathway reporter gene in the presence and absence of a test agent;

5 b) determining whether said test agent inhibits the induction of the NF- $\kappa$ B pathway reporter gene by CARD-4; and

c) identifying a compound that inhibits the induction of the NF- $\kappa$ B pathway reporter gene by CARD-4.

10 18. A method for identifying a compound that inhibits the enhancement of caspase 9 activity by a CARD-4 protein comprising a CARD-4 domain comprising the steps of:

a) incubating a recombinant cell line expressing caspase 9 and CARD-4 in the presence and absence of a  
15 test agent;

b) determining whether said test agent inhibits caspase 9 activity; and

c) identifying a compound that inhibits the  
20 enhancement of caspase 9 activity by a CARD-4 protein.

19. The method of claim 18 further comprising:

a) incubating a recombinant cell line expressing caspase 9 and CARD-4 and a beta-galactosidase expression vector in the presence and absence of a test agent;

25 b) determining whether the presence of said test agent increases the proportion of cells that stain positive for beta-galactosidase; and

c) identifying a compound that inhibits the enhancement of caspase 9 activity by a CARD-4 protein by  
30 identifying a compound that increases the proportion of cells that stain positive for beta-galactosidase.

- 142 -

20. The method of claim 18, wherein the CARD-4 protein comprising a CARD-4 domain is selected from the group consisting of:

- a) a polypeptide comprising the amino acid  
5 sequence of SEQ ID NO:8;
- b) a polypeptide comprising the amino acid sequence of SEQ ID NO:39;
- c) a polypeptide comprising the amino acid sequence of SEQ ID NO:41; and
- 10 d) a polypeptide comprising the amino acid sequence of SEQ ID NO:43.

21. A method for identifying a compound that inhibits the enhancement of caspase 9 activity by a CARD-3 protein comprising a CARD-3 domain comprising the  
15 steps of:

- a) incubating a recombinant cell line expressing caspase 9 and CARD-3 in the presence and absence of a test agent;
- b) determining whether said test agent inhibits  
20 caspase 9 activity; and
- c) identifying a compound that inhibits the enhancement of caspase 9 activity by a CARD-3 protein.

22. The method of claim 21 further comprising:

- a) incubating a recombinant cell line expressing  
25 caspase 9 and CARD-3 and a beta-galactosidase expression vector in the presence and absence of a test agent;
- b) determining whether the presence of said test agent increases the proportion of cells that stain positive for beta-galactosidase; and

- 143 -

c) identifying a compound that inhibits the enhancement of caspase 9 activity by a CARD-3 protein by identifying a compound that increases the proportion of cells that stain positive for beta-galactosidase.

CCACGCGTCCGGTCAGCTCTGGTTCCGAGAAGCAGCGGGCTGGCGTGGGCCATCCGGGAATGGGC  
GCCCTCGTGACCTAGTGTTGCGGGGCAAAAAGGGTCTTSCCGGCCTCGCTCGTGACAGGGGCGTAT  
TTGGGCGCCTGAGCGCGGCGTGGGAGCCTTSGGAGCCGCGCGCAGCAGGGGGCACACCCGGAACCG  
GCCTGAGCGCCCCGGGACCATGAACGGGGAGGCCATCTGCAGCGCCCTGCCACCATTCCCTACCA  
CAAACCTCGCCGACCTGCGCTACCTGAGCCGCGGCGCCTCTGGCACTGTGTCTCGCCCGCGCACG  
CAGACTGGCGCGTCCAGGTGGCCGTGAAGCACCTGCACATCCACACTCCGCTGCTCGACAGTGAA  
AGAAAGGATGTCTTAAGAGAAGCTGAAATTTTACACAAAGCTAGATTTAGTTACATTCTTCCAAT  
TTTGGGAATTTGCAATGAGCCTGAATTTTGGGAATAGTTACTGAATACATGCCAAATGGATCAT  
TAAATGAACTCCTACATAGGAAAACCTGAATATCCTGATGTTGCTTGGCCATTGAGATTTCCATC  
CTGCATGAAATTGCCCTTGGTGTAAATTACCTGCACAATATGACTCCTCCTTTACTTCATCATGA  
CTTGAAGACTCAGAATATCTTATTGGACAATGAATTTTCATGTTAAGATTGCAGATTTTGGTTTAT  
CAAAGTGGCGCATGATGTCCCTCTCACAGTCACGAAGTAGCAAATCTGCACCAGAAGGAGGGACA  
ATTATCTATATGCCACCTGAAAACCTATGAACCTGGACAAAAATCAAGGGCCAGTATCAAGCACGA  
TATATATAGCTATGCAGTTATCACATGGGAAGTGTTATCCAGAAAACAGCCTTTTGAAGATGTCA  
CCAATCCTTTGCAGATAATGTATAGTGTGTACAAAGGACATCGACCTGTTATTAATGAAGAAAGT  
TTGCCATATGATATACCTCACCGAGCACGTATGATCTCTCTAATAGAAAGTGGATGGGCACAAAA  
TCCAGATGAAAGACCATCTTTCTTAAATGTTTAAATAGAAGTTGAACCAAGTTTGGAGAACATTTG  
AAGAGATAACTTTTTCTTGAAGCTGTTATTCAGCTAAAGAAAACAAAGTTACAGAGTGTTCAGGT  
GCCATTCACCTATGTGACAAGAAGAAAATGGAATTATCTCTGAACATACCTGTAAATCATGGTCC  
ACAAGAGGAATCATGTGGATCCTCTCAGCTCCATGAAAATAGTGGTTCTCCTGAAACTTCAAGGT  
CCCTGCCAGCTCCTCAAGACAATGATTTTTTATCTAGAAAAGCTCAAGACTGTTATTTTATGAAG  
CTGCATCACTGTCTTGGAAATCACAGTTGGGATAGCACCATTCTGGATCTCAAAGGGCTGCATT  
CTGTGATCACAAAGACCATTCCATGCTCTTCAGCAATAATAAATCCACTCTCAACTGCAGGAACT  
CAGAACGTCTGCAGCCTGGTATAGCCCAGCAGTGGATCCAGAGCAAAAGGGAAGACATTGTGAAC  
CAAATGACAGAAGCCTGCCTTAACCAAGTCGCTAGATGCCCTTCTGTCCAGGGACTTGATCATGAA  
AGAGGACTATGAACTTGTTAGTACCAAGCCTACAAGGACCTCAAAAGTCAGACAATTACTAGACA  
CTACTGACATCCAAGGAGAAGAATTTGCCAAAGTTATAGTACAAAATTGAAAGATAACAAACAA  
ATGGGTCTTCAGCCTTACCCGAAATACTTGTGGTTTCTAGATCACCATCTTTAAATTTACTTCA  
AAATAAAAGCATGTAAGTGACTGTTTTTCAAGAAGAAATGTGTTTCATAAAAGGATATTTATAAA  
AA (SEQ ID NO:1)

FIG. 1

Met	Asn	Gly	Glu	Ala	Ile	Cys	Ser	Ala	Leu	Pro	Thr	Ile	Pro	Tyr	His	Lys	Leu	Ala	Asp	20
Leu	Arg	Tyr	Leu	Ser	Arg	Gly	Ala	Ser	Gly	Thr	Val	Ser	Ser	Ala	Arg	His	Ala	Asp	Trp	40
Arg	Val	Gln	Val	Ala	Val	Lys	His	Leu	His	Ile	His	Thr	Pro	Leu	Leu	Asp	Ser	Gln	Arg	60
Lys	Asp	Val	Leu	Arg	Glu	Ala	Glu	Ile	Leu	His	Lys	Ala	Arg	Phe	Ser	Tyr	Ile	Leu	Pro	80
Ile	Leu	Gly	Ile	Cys	Asn	Glu	Pro	Glu	Phe	Leu	Gly	Ile	Val	Thr	Glu	Tyr	Met	Pro	Asn	100
Gly	Ser	Leu	Asn	Glu	Leu	Leu	His	Arg	Lys	Thr	Glu	Tyr	Pro	Asp	Val	Ala	Trp	Pro	Leu	120
Arg	Phe	Arg	Ile	Leu	His	Glu	Ile	Ala	Leu	Gly	Val	Asn	Tyr	Leu	His	Asn	Met	Thr	Pro	140
Pro	Leu	Leu	His	His	Asp	Leu	Lys	Thr	Gln	Asn	Ile	Leu	Leu	Asp	Asn	Gln	Phe	His	Val	160
Lys	Ile	Ala	Asp	Phe	Gly	Leu	Ser	Lys	Trp	Arg	Met	Met	Ser	Leu	Ser	Gln	Ser	Arg	Ser	180
Ser	Lys	Ser	Ala	Pro	Glu	Gly	Gly	Thr	Ile	Ile	Tyr	Met	Pro	Pro	Glu	Asn	Tyr	Gln	Pro	200
Gly	Gln	Lys	Ser	Arg	Ala	Ser	Ile	Lys	His	Asp	Ile	Tyr	Ser	Tyr	Ala	Val	Ile	Thr	Trp	220
Glu	Val	Leu	Ser	Arg	Lys	Gln	Pro	Phe	Glu	Asp	Val	Thr	Asn	Pro	Leu	Gln	Ile	Met	Tyr	240
Ser	Val	Ser	Gln	Gly	His	Arg	Pro	Val	Ile	Asn	Glu	Glu	Ser	Leu	Pro	Tyr	Asp	Ile	Pro	260
His	Arg	Ala	Arg	Met	Ile	Ser	Leu	Ile	Glu	Ser	Gly	Trp	Ala	Gln	Asn	Pro	Asp	Gln	Arg	280
Pro	Ser	Phe	Leu	Lys	Cys	Leu	Ile	Glu	Leu	Glu	Pro	Val	Leu	Arg	Thr	Phe	Glu	Gln	Ile	300
Thr	Phe	Leu	Glu	Ala	Val	Ile	Gln	Leu	Lys	Lys	Thr	Lys	Leu	Gln	Ser	Val	Ser	Ser	Ala	320
Ile	His	Leu	Cys	Asp	Lys	Lys	Lys	Met	Glu	Leu	Ser	Leu	Asn	Ile	Pro	Val	Asn	His	Gly	340
Pro	Gln	Glu	Glu	Ser	Cys	Gly	Ser	Ser	Gln	Leu	His	Glu	Asn	Ser	Gly	Ser	Pro	Gln	Thr	360
Ser	Arg	Ser	Leu	Pro	Ala	Pro	Gln	Asp	Asn	Asp	Phe	Leu	Ser	Arg	Lys	Ala	Gln	Asp	Cys	380
Tyr	Phe	Met	Lys	Leu	His	His	Cys	Pro	Gly	Asn	His	Ser	Trp	Asp	Ser	Thr	Ile	Ser	Gly	400
Ser	Gln	Arg	Ala	Ala	Phe	Cys	Asp	His	Lys	Thr	Ile	Pro	Cys	Ser	Ser	Ala	Ile	Ile	Asn	420
Pro	Leu	Ser	Thr	Ala	Gly	Asn	Ser	Glu	Arg	Leu	Gln	Pro	Gly	Ile	Ala	Gln	Gln	Trp	Ile	440
Gln	Ser	Lys	Arg	Glu	Asp	Ile	Val	Asn	Gln	Met	Thr	Glu	Ala	Cys	Leu	Asn	Gln	Ser	Leu	460
Asp	Ala	Leu	Leu	Ser	Arg	Asp	Leu	Ile	Met	Lys	Glu	Asp	Tyr	Glu	Leu	Val	Ser	Thr	Lys	480
Pro	Thr	Arg	Thr	Ser	Lys	Val	Arg	Gln	Leu	Leu	Asp	Thr	Thr	Asp	Ile	Gln	Gly	Glu	Glu	500
Phe	Ala	Lys	Val	Ile	Val	Gln	Lys	Leu	Lys	Asp	Asn	Lys	Gln	Met	Gly	Leu	Gln	Pro	Tyr	520
Pro	Glu	Ile	Leu	Val	Val	Ser	Arg	Ser	Pro	Ser	Leu	Asn	Leu	Leu	Gln	Asn	Lys	Ser	Met	540

(SEQ ID NO:2)

FIG. 2

TTTTTATGGG AATCGCAGCT TGGAGAGAG AGARCAATTC CAGAAWTAAA TTGRAATTGA  
AGATTTAACC AATGTTGTTT TAAATATTC TAACTTCAAA GAATGATGCC AGAACTTNAA  
AAGGGRCTGC GCAGAGTAGC AGGGGCCCTG GAGGGCGCGG CCTGAATCCT GATTGCCCTT  
CTGCTGAGAG GACACACGCA GCTGAAGATG AATTGGGAA AAGTAGCCGC TTGCTACTTT  
AACTATGGAA GAGCAGGGCC ACAGTGAGAT GGAAATAATC CCATCAGAGT CTCACCCCA  
CATTCAATTA CTGAAAAGCA ATCGGGAAC TCTGGTCACT CACATCCGCA ATACTCAGTG  
TCTGGTGGAC AACTTGCTGA AGAATGACTA CTTCTCGGCC GAAGATGCGG AGATTGTGTG  
TGCCTGCCCC ACCCAGCCTG ACAAGGTCCG CAAAATTCTG GACCTSGTAC AGAGCAAGGG  
CGAGGAGGTG TCCGAGTTCT TCCTCTACTT GCTCCAGCAA CTCGCAGATG CCTACGTGGA  
CCTCAGGCCT TGGCTGCTGG AGATCGGCTT CTCCCTTCC CTGCTCACTC AGAGCAAAGT  
CGTGGTCAAC ACTGACCCAG TGAGCAGGTA TACCCAGCAG CTGCGACACC ATCTGGGCGG  
TGACTCCAAG TTCGTGCTGT GCTATGCCCA GAAGGAGGAG CTGCTGCTGG AGGAGATCTA  
CATGGACACC ATCATGGAGC TGGTTGGCTT CAGCAATGAG AGCCTGGGCA GCCTGAACAG  
CCTGGCCTGC CTCCTGGACC ACACCACCGG CATCCTCAAT GAGCAGGGTG AGACCATCTT  
CATCCTGGGT GATGCTGGGG TGGGCAAGTC CATGCTGCTA CAGCGGCTGC AGAGCCTCTG  
GGCCACGGGC CGGCTAGACG CAGGGGTCAA ATTCTTCTTC CACTTTCGCT GCCGCATGTT  
CAGCTGCTTC AAGGAAAGTG ACAGGCTGTG TCTGCAGGAC CTGCTCTTCA AGCACTACTG  
CTACCCAGAG CGGGACCCCG AGGAGGTGTT TGCCTTCCTG CTGCGCTTCC CCCACGTGGC  
CCTCTTCACC TTCGATGGCC TGGACGAGCT GCCTCGGAC TTGGACCTGA GCCGCGTGCC  
TGACAGCTCC TGCCCTGGG AGCCTGCCCA CCCCCTGGTC TTGCTGGCCA ACCTGCTCAG  
TGGGAAGCTG CTCAAGGGGG CTAGCAAGCT GCTCAGACC CGCACAGGCA TCGAGGTCCC  
GCGCCAGTTC CTGCGGAAGA AGGTGCTTCT CCGGGGCTTC TCCCCAGCC ACCTGCGCGC  
CTATGCCAGG AGGATGTTCC CCGAGCGGSC CTTGCAGGAC CGCCTGCTGA GCCAGCTGGA  
GGCCAAACCC AACCTCTGCA GCCTGTGCTC TGTGCCCTC TTCTGCTGGA TCATCTTCCG  
GTGCTTCCAG CACTTCCGTG CTGCCTTTGA AGGCTCACC CAGCTGCCCC ACTGCACGAT  
GACCCTGACA GATGTCTTCC TCCTGGTCAC TGAGGTCCAT CTGAACAGGA TGCAGCCCAG  
CAGCCTGGTG CAGCGGAACA CACGCAGCCC AGTGGAGACC CTCCACGCCG GCCGGGACAC  
TCTGTGCTCG CTGGGGCAGG TGGCCACCG GGGCATGGAG AAGAGCCTCT TTGTCTTCAC  
CCAGGAGGAG GTGCAGGCCT CCGGGCTGCA GGAGAGAGAC ATGCAGCTGG GCTTCCTGCG  
GGCTTTGCCG GAGCTGGGCC CCGGGGGTGA CCAGCAGTCC TATGAGTTTT TCCACCTCAC  
CCTCCAGGCC TTCTTTACAG CCTTCTTCCT CGTGTGGAC GACAGGGTGG GCACTCAGGA  
GCTGCTCAGG TTCTTCCAGG AGTGGATGCC CCCTGCGGGG GCAGCGACCA CGTCTCTGTA

FIG. 3 (page 1 of 2)

TCCTCCCTTC CTCCTGTTCC AGTGCTTSCA GGGCAGTGGT CCGGCGCGGG AAGACCTCTT  
CAAGAACAAG GATCCTTTCC AGTTCACCAA CCTCTTCCTG TCGGGGCTGT TGTCCAAAGC  
CAAACAGAAA CTCCTGCGGC ATCTGGTGCC CGCGGCAGCC CTGAGGAGAA AGCGCAAGGC  
CCTGTGGGCA CACCTGTTTT CCAGCCTSCG GGGCTACCTG AAGAGCCTGC CCCGCGTTCA  
GGTCGAAAGC TTCAACCAGG TGCAGGCCAT GCGCACGTTT ATCTGGATGC TCGGCTGCAT  
CTACGAGACA CAGAGCCAGA AGGTGGGGCA GCTGGCGGCC AGGGGCATCT GCGCCAACTA  
CCTCAAGCTG ACCTACTGCA ACGCCTGCTC GGCCGACTGC AGCGCCCTCT CCTTCGTCCT  
GCATCACTTC CCCAAGCGGC TGGCCCTAGA CCTAGACAAC AACAATCTCA ACGACTACGG  
CGTGCGGGAG CTGCAGCCCT GCTTCAGCCG CCTCACTGTT CTCAGACTCA GCGTAAACCA  
GATCACTGAC GGTGGGGTAA AGGTGCTAAG CGAAGAGCTG ACCAAATACA AAATTGTGAC  
CTATTTGGGT TTATACAACA ACCAGATCAC CGATGTGCGA GCCAGGTACG TCACCAAAAT  
CCTGGATGAA TGCAAAGGCC TCACGCATCT TAACTGGGA AAAAACAAAA TAACAAGTGA  
AGGAGGGAAG TATCTCGCCC TGGCTGTGAA GAACAGCAAA TCAATCTCTG AGGTTGGGAT  
GTGGGGCAAT CAAGTTGGGG ATGAAGGAGC AAAAGCCTTC GCAGAGGCTC TGCGGAACCA  
CCCCAGCTTG ACCACCTGA GTCTTGCGTC CAACGGCATC TCCACAGAAG GAGGAAAGAG  
CCTTGCGAGG GCGCTGCAGC AGAACACGTC TCTAGAAATA CTGTGGCTGA CCCAAAATGA  
ACTCAACGAT GAAGTGGCAG AGAGTTTGGC AGAAATGTTG AAAGTCAACC AGACGTTAAA  
GCATTTATGG CTTATCCAGA ATCAGATCAC AGCTAAGGGG ACTGCCCAGC TGGCAGATGC  
GTTACAGAGC AACACTGGCA TAACAGAGAT TTGCCTAAAT GGAAACCTGA TAAAACCAGA  
GGAGGCCAAA GTCTATGAAG ATGAGAAGCG GATTATCTGT TTCTGAGAGG ATGCTTTTCT  
GTTATGTTGG TTTTTCCTT GAGGCTCTAG CAGCAAATGC CACTCTGGGC AGTCTTTTGT  
GTCAGTGTCT TAAAGGGGCC TCGCGAGGCG GGAATATCAG GAGTCCACTG CCTYCATGAT  
GCAAGCCAGC TTCCTGTGCA GAAGGTCTGG TCGGCAAACT CCCTAAGTAC CCGCTACAAT  
TCTGCAGAAA AAGAATGTGT CTTGCGAGCT GTTGTAGTTA CAGTAAATAC ACTGTGAAGA  
GAAAAAAAAA ACGGACGCGT GG (SEQ ID NO:7)

FIG. 3 (page 2 of 2)



MEEQGHSEMEIIPSESHPHIQLLKSNRELLVTHIRNTQCLVDNLLKNDYFSAEDAEIVCACPTQP  
DKVRKILDVQSKGEEVSEFFLYLLQQLADAYVDLRPWLLLEIGFSPSLLTQSKVVVNTDPVSRYT  
QQLRHHLGRDSKFVLCYAQKEELLLLEETIMELVGFSNESLGSLSLACLDDHTTGILNEQG  
ETIFILGDAGVGKSMMLLQRLQSLWATGRLDAGVKFFFHFRCRMFSCKESDRLCLQDLLFKHYCY  
FERDPFEEVFAFLLRFPHPVALFTFDGLDELHSDLDLSRVPDSSCPWEPAPLVLNLLSGKLLKG  
ASKLLTARTGIEVPRQFLRKKVLLRGFSPSHLRAYARRMFPERALQDRLLSQLEANPNLCSLCSV  
PLFCWIIIFRCFQHFRAAFEGSPQLPDCTMTLTDVFLLVTEVHLNRMQPSSLVQRNTRSPVETLHA  
GRDTLCSLGQVAHRGMEKSLFVFTQEEVQASGLQERDMQLGFLRALPELGPGGDQOSYEFFHLTL  
QAFFTAFFLVLDLDRVGTCELLRFFQEWMPAGAAATTSCYPPFLPFQCLQSGPAREDLFKNKDH  
QFTNLFLCGLLSKAKQKLLRHLVPAAALRRKRKALWAHLFSSLRGYLKSLPRVQVESFNQVQAMP  
TFIWMLRCIYETQSQKVGQLAARGICANYLKLTYCNACSADCSALSFVLHHFPKRLALDLDNNNL  
NDYGVRELQPCFSRLTVLRLSVNQITDGGVKVLSEELTKYKIVTYLGLYNNQITDVGARYVTKIL  
DECKGLTHLKLGNKITSEGGKYLALAVKNSKSI SEVGMWGNQVGDGAKAFAEALRNHPSLTTL  
SLASNGISTEGGKSLARALQQNTSLEILWLTQNELNDEVAESLAEMLKVNQTLKHLWLIQNQITA  
KGTAQLADALQSNTGITEICLNGNLIKPEEAKVYEDEKRIICF (SEQ ID NO:8)

FIG. 4

TACGCGTCCGACTTGGCTGAAGAATGACTACTTCTCGGCCGAAGATGCGGAGATTGTGT  
BTGCCTGCCCCACCCAGCCTGACAAGGTCCGCAAAATTCTGGACCTGGTACAGAGCAAG  
GGCGAGGAGGTGTCCGAGTTCTTCTCTACTTGCTCCAGCAACTCGCAGATGCCTACGT  
GGACCTCAGGCCTTGGCTGCTGGAGATCGGCTTCTCCCTTCCCTGCTCACTCAGAGCA  
AAGTCGTGGTCAACACTGACCCAGTGAGCAGGTATACCCAGCAGCTGCGACACCATCTG  
GGCCGTGACTCCAAGTTCTGTGCTGTGCTATGCCAGAAAGGAGGAGCTGCTGCTGGAGGA  
GATCTACATGGACACCATCATGGAGCTGGTTGGCTTCAGCAATGAGAGCCTGGGCAGCC  
TGAACAGCCTGGCCTGCCTCCTGGACCACACCACCGGCATCCTCAATGAGCAGGGTGAG  
ACCATCTTCATCCTGGGTGATGCTGGGGTGGGCAAGTCCATGCTGCTACAGCGGCTGCA  
GAGCCTCTGGGCCACGGGCCGGCTAGACGCAGGGGTCAAATTCTTCTTCCACTTTTCGCT  
GCCGCATGTTTCAAGTGTGCTTCAAGGAAAGTGACAGGCTGTGTCTGCAGGACCTGCTCTTC  
AAGCACTACTGCTACCCAGAGCGGGACCCCGAGGAGGTGTTTGCCTTCTGCTGCGCTT  
CCCCACGTGGCCCTCTTCACTTTCGATGGCCTGGACGAGCTGCACTCGGACTTGGACC  
TGAGCCGCGTGCCTGACAGCTCCTGCCCTGGGAGCCTGCCCACCCCTGGTCTTGCTG  
GCCAACCTGCTCAGTGGGAAGCTGCTCAAGGGGGCTAGCAAGCTGCTCACAGCCCGCAC  
AGGCATCGAGGTCCCGCGCCAGTTCTCGGGAAGAAGGTGCTTCTCCGGGGCTTCTCCC  
CCAGCCACCTGCGCGCTATGCCAGGAGGTGTTCCCGAGCGGGCCCTGCAGGACCGC  
CTGCTGAGCCAGCTGGAGGCCAACCCCAACCTCTGCAGCCTGTGCTCTGTGCCCCCTCTT  
CTGCTGGATCATCTTCCGGTGCTTCCAGCACTTCCGTGCTGCCTTTGAAGGCTCACCAC  
AGCTGCCCCGACTGCACGATGACCCTGACAGATGTCTTCTCCTGGTCACTGAGGTCCAT  
CTGAACAGGATGCAGCCCAGCAGCCTGGTGCAGCGGAACACACGCAGCCCAGTGGAGAC  
CCTCCACGCCGCGCGGACACTCTGTGCTCGCTGGGGCAGGTGGCCCACCGGGGCATGG  
AGAAGAGCCTCTTTGTCTTCACCAGGAGGAGGTGCAGGCCTCCGGGCTGCAGGAGAGA  
GACATGCAGCTGGGCTTCTTGCGGGCTTTGCCGGAGCTGGGCCCGGGGGTGACCAGCA  
GTCCTATGAGTTTTTCCACCTCAGCCTCCTCACCTGTAAACTGGGATCCAGTATAGA  
CTTTGGAAATCAGTAGACACCATATGCTTCAAAAACAGGGGCTATTAAATGACATCA  
GGAGCCAGAAAGTCTCATGGCTGTGCTTTCTCTTGAAGTTTATACAACAACAGATCAC  
CGATGTGCGAGCCAGACTGGGAAAAAACAATAACAAGTGAAGGAGGGAAGTATCTCG  
CCCTGGCTGTGAAGAACAGCAAATCAATCTCTGAGGTGGGATGTGGGGCAATCAAGTT  
GGGGATGAAGGAGCAAAGCCTTCGCAGAGGCTCTGCGGAACCAACCCAGCTTGACCAC  
CCTGAGTCTTGCGTCCAACGGCATCTCCACAGAAGGAGGAAAGAGCCTTGCGAGGGCCC  
TGCAGCAGAACACGTCTCTAGAAATACTGTGGCTGACCCAAAATGAACTCAACGATGAA  
BTGGCAGAGAGTTTGGCAGAAATGTTGAAAGTCAACCAGACGTTAAAGCATTATGGCT  
TATCCAGAATCAGATCACAGTCTTTGTGTGCTAGTGTCTTAAAGGGGCTGCGCAGGCGG  
GACTATCAGGAGTCCACTGCCTCCATGATGCAAGCCAGCTTCTGTGCAGAAAGTCTGG  
TCGGCAAACCTCCCTAAGTACCCGCTACAATTCTGCAGAAAAAGAATGTGTCTTGCGAGC  
TGTGTAGTTACAGTAAATACACTGTGAAGAGACTTTATTGCCTATTATAATTATTTT  
ATCTGAAGCTAGAGGAATAAAGCTGTGAGCAAACAGAGGAGGCCAGCCTCACCTCATTC  
CAACACCTGCCATAGGGACCAACGGGAGCGAGTTGGTCACCGCTCTTTTCATTGAAGAG  
TTGAGGATGTGGCACAAGTTGGTGCCAAGCTTCTTGAATAAAACGTGTTTGATGGATT  
AGTATTATACCTGAAATATTTTCTTCTCCTTCTCAGCACTTCCCATGTATTGATACTGGT  
CCCACTTCACAGCTGGAGACACCGGAGTATGTGCAGTGTGGGATTGACTCCTCCAAGG  
TTTTGTGGAAAGTTAATGTCAAGGAAAGGATGCACCACGGGCTTTTAATTTTAATCCTG  
GAGTCTCACTGTCTGCTGGCAAAGATAGAGAATGCCCTCAGCTCTTAGCTGGTCTAAGA  
ATGACGATGCCTTCAAAATGCTGCTTCCACTCAGGGCTTCTCCTCTGCTAGGCTACCCCT  
CCTCTAGAAGGCTGAGTACCATGGGCTACAGTGTCTGGCCTTGGGAAGAAGTGATTCTG  
TCCCTCCAAGGAAATAGGGCATGGCTTGCCCTGTGGCCTGGCCATCCAATGGCTGCT  
TTGTCTCCCTTACCTCGTGAAGAGGGGAAGTCTCTTCTGCTGCCCTCCAAGCAGCTGAAG  
GCTGACTAAACGGGCGCCAAGACTCAGGGGATCGGCTGGGAACTGGGCCAGCAGAGCAT  
GTTGGACACCCCCCACCATGGTGGGCTTGTGGTGGCTGCTCCATGAGGGTGGGGGTGAT  
ACTACTAGATCACTTGTCTCTTGGCAGCTCATTTGTTAATAAAATACTGAAAACACAA  
AA  
AAAAAAAAAAAAA (SEQ ID NO:25)

FIG. 5



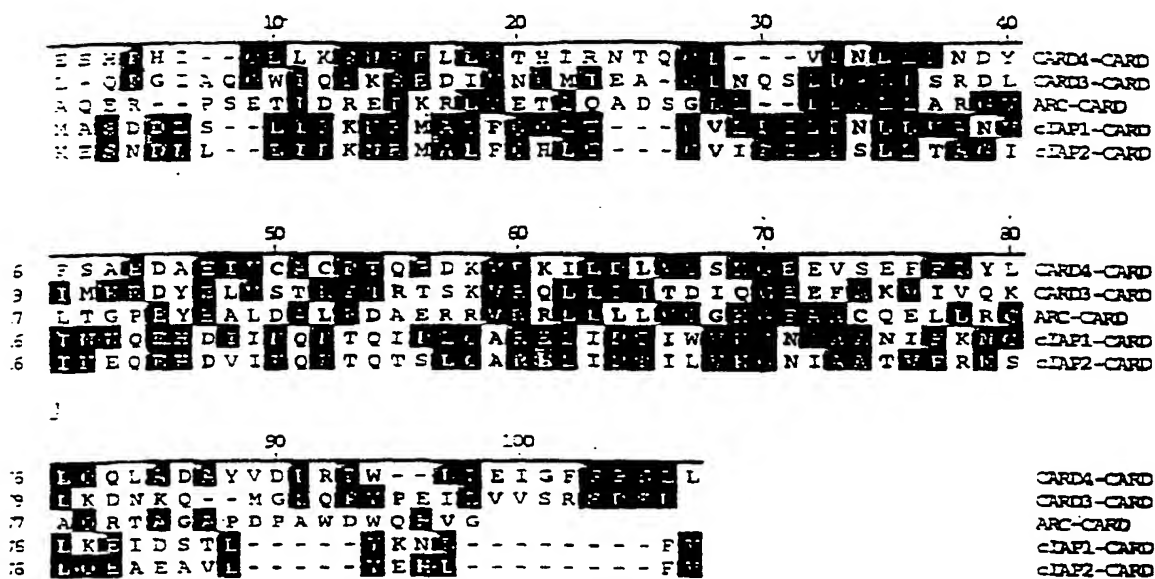


FIG. 7

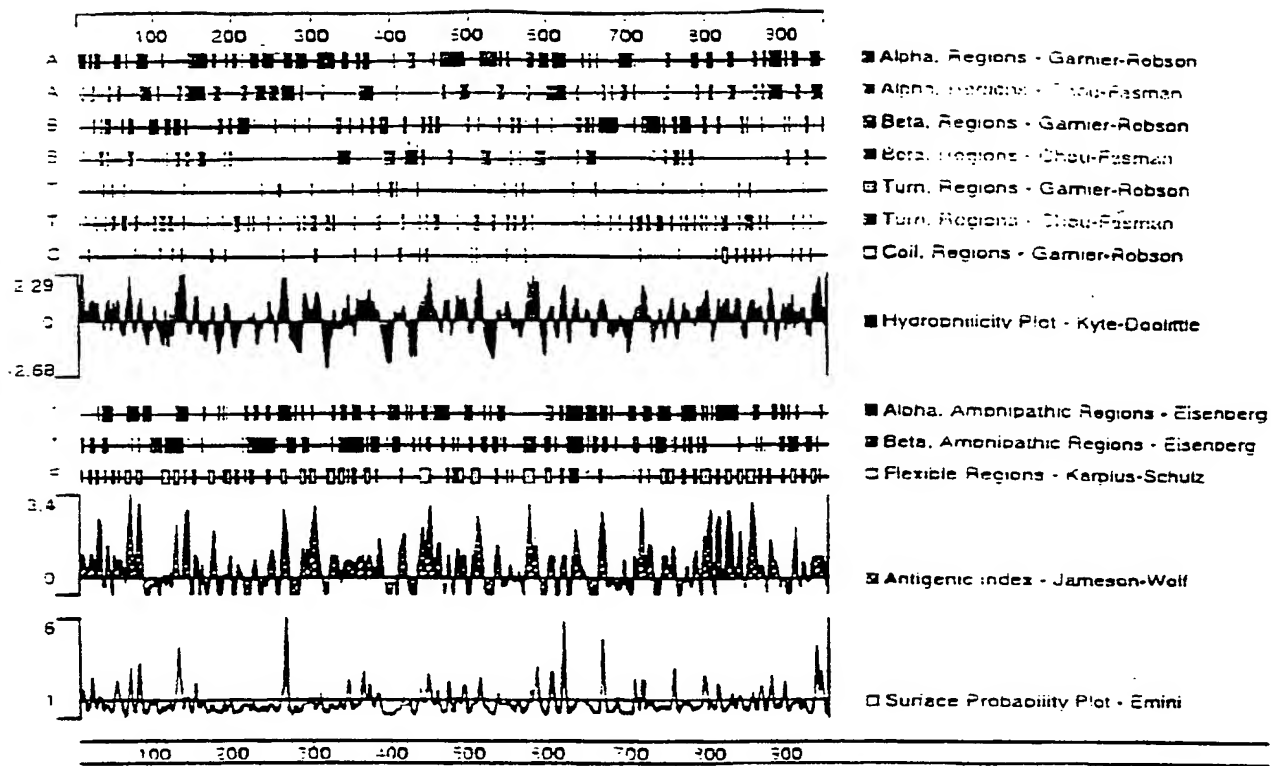


FIG. 8

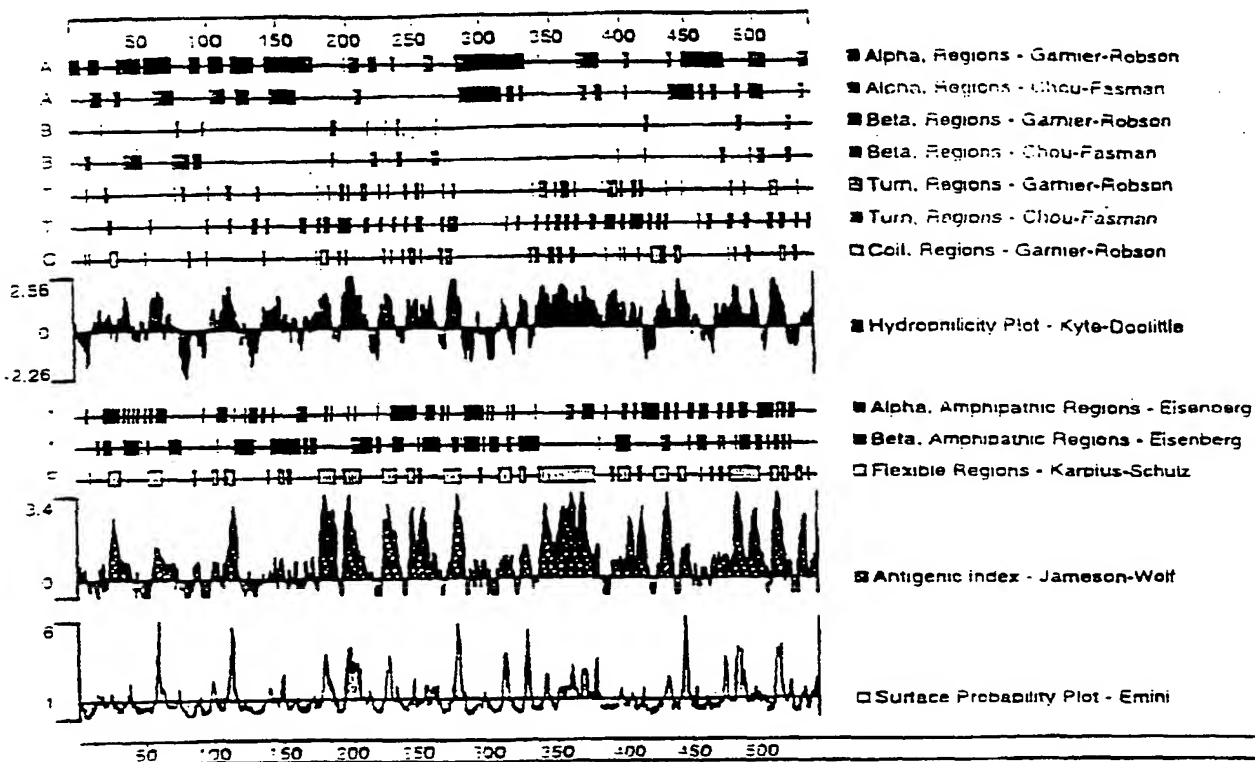


FIG. 9

CCCGCGTCCGCGTCCCCGGACCATGGCGCTCTCCGGGCTCTTCTCTAGCTCTCAGCGGCT  
GCGAAGTCTGTNAACCTGGTGGCCAAGTGATTGTAAGTCAGGAGACTTTCCTTCGGTTTC  
TGCCTTTGATGGCAAGAGGTGGAGATTGTGGCGGCGATTACAGAAAACATCTGGGAAGAC  
AAGTTGCTGTTTTTATGGGAATCGCAGGCTTGGAAGAGACAGAAGCAATTCAGAAATAA  
ATTGGAAATTGAAGATTTAAACAATGTTGTTTTAAATATTCTAACTTCAAAGAATGATG  
CCAGAACTTAAAAAGGGGCTGCGCAGAGTAGCAGGGGCCCTGGAGGGCGCGGCCTGAAT  
CCTGATTGCCCTTCTGCTGAGAGGACACACGCAGCTGAAGATGAATTTGGGAAAAGTAGC  
CGCTTGCTACTTTAACTATGGAAGAGCAGGGCCACAGTGAGATGGAAATAATCCCATCAG  
AGTCTCACCCCCACATTCAATTACTGAAAAGCAATCGGGAACTTCTGGTCACTCACATCC  
GCAATACTCAGTGTCTGGTGGACAACCTTGCTGAAGAATGACTACTTCTCGGCCGAAGATG  
CGGAGATTGTGTGTGCCTGCCCCACCCAGCCTGACAAGGTCCGCAAATTTCTGGACCTGG  
TACAGAGCAAGGGCGAGGAGGTGTCCGAGTTCTTCCTCTACTTGCTCCAGCAACTCGCAG  
ATGCCTACGTGGACCTCAGGCCTTGGCTGCTGGAGATCGGCTTCTCCCCCTTCCCTGCTCA  
CTCAGAGCAAAGTCGTGGTCAACACTGACCCAGTGAGCAGGTATACCCAGCAGCTGCGAC  
ACCATCTGGGCCGTGACTCCAAGTTCGTGCTGTGCTATGCCCAGAAGGAGGAGCTGCTGC  
TGGAGGAGATCTACATGGACACCATCATGGAGCTGGTTGGCTTCAGCAATGAGAGCCTGG  
GCAGCCTGAACAGCCTGGCCTGCCTCCTGGACCACACCACCGGCATCCTCAATGAGCAGG  
CTGCTTCAAGGAAAGTGACAGGCTGTGTCTGCAGGACCTGCTCTTCAAGCACTACTGCTA  
CCCAGAGCGGGACCCCGAGGAGGTGTTTGCCTTCTTGCTGCGCTTCCCCCACGTGGCCCT  
CTTCACCTTCGATGGCCTTGACGAGCTGCACTCGGACTTGACCTGAGCCGCGTGCCTGA  
CAGCTCCTGCCCCCTGGGAGCCTGCCCCCCCCCTGGTCTTGCTGGCCAACCTGCTCAGTGG  
GAAGCTGCTCAAGGGGGCTAGCAAGCTGCTCACAGCCCGCACAGGCATCGAGGTCCCGCG  
CCAGTTCTGCGGAAGAAGGTGCTTCTCCGGGGCTTCTCCCCAGCCACCTGCGCGCCTA  
TGCCAGGAGGATGTTCCCCGAGCGGGCCCTGCAGGACCGCCTGCTGAGCCAGCTGGAGGC  
CAACCCCAACCTCTGCAGCCTGTGCTCTGTGCCCCCTTCTGCTGGATCATCTTCCGGTG  
CTTCCAGCACTTCCGTGCTGCCTTTGAAGGCTCACACAGCTGCCCCGACTGCACGATGAC  
CCTGACAGATGTCTTCTCCTGGTCACTGAGGTCCATCTGAACAGGATGCAGCCCAGCAG  
CCTGGTGCAGCGGAACACACGCAGCCCAGTGGAGACCCTCCACGCCGGCCGGGACACTCT  
GTGCTCGCTGGGGCAGGTGGCCCACCGGGGCATGGAGAAGAGCCTCTTTGTCTTACCCA  
GGAGGAGGTGCAGGCCTCCGGGCTGCAGGAGAGAGACATGCAGCTGGGCTTCTGCGGGC  
TTTGCCGGAGCTGGGGCCCCGGGGGTGACCAGCAGTCCTATGAGTTTTTCCACCTCACCT

FIG. 10 (Page 1 of 3)

CCAGGCCTTCTTTACAGCCTTCTTCCTCGTGCTGGACGACAGGGTGGGCACTCAGGAGCT  
GCTCAGGTTCTTCCAGGAGTGGATGCCCCCTGCGGGGGCAGCGACCACGTCCTGCTATCC  
TCCCTTCTTCCCGTTCCAGTGCCTGCAGGGCAGTGGTCCGGCGCGGGAAGACCTCTTCAA  
GAACAAGGATCACTTCCAGTTCACCAACCTCTTCCTGTGCGGGCTGTTGKCCAAAGCCAA  
ACAGAAACTCCTGCGGCATCTGGTGCCCGCGGCAGCCCTGAGGAGAAAGCGCAAGGCCCT  
GTGGGCACACCTGTTTTTCCAGCCTGCGGGGCTACCTGAAGAGCCTGCCCCGCGTTCAGGT  
CGAAAGCTTCAACCAGGTGCAGGCCATGCCCACGTTTCATCTGGATGCTGCGCTGCATCTA  
CGAGACACAGAGCCAGAAGGTGGGGCAGCTGGCGGCCAGGGGCATCTGCGCCAACTACCT  
CAAGCTGACCTACTGCAACGCCTGCTCGGCCGACTGCAGCGCCCTCTCCTTCGTCCTGCA  
TCACTTCCCCAAGCGGCTGGCCCTAGACCTAGACAACAACAATCTCAACGACTACGGCGT  
GCGGGAGCTGCAGCCCTGCTTCAGCCGCCTCACTGTTCTCAGACTCAGCGTAAACCAGAT  
CACTGACGGTGGGGTAAAGGTGCTAAGCGAAGAGCTGACCAAATACAAAATTGTGACCTA  
TTTGGGTTTATACAACAACCAGATCACCGATGTGCGAGCCAGGTACGTCACCAAAAATCCT  
GGATGAATGCAAAGGCCTCACGCATCTTAAACTGGGAAAAAACAAAATAACAAGTGAAGG  
AGGGAAGTATCTCGCCCTGGCTGTGAAGAACAGCAAATCAATCTCTGAGGTTGGGATGTG  
GGGCAATCAAGTTGGGGATGAAGGAGCAAAAGCCTTCGCAGAGGCTCTGCGGAACCAACC  
CAGCTTGACCACCCTGAGTCTTGCGTCCAACGGCATCTCCACAGAAGGAGGAAAGAGCCT  
TGCGAGGGCCCTGCAGCAGAACACGTCTCTAGAAATACTGTGGCTGACCCAAAATGAACT  
CAACGATGAAGTGGCAGAGAGTTTGGCAGAAATGTTGAAAGTCAACCAGACGTTAAAGCA  
TTTATGGCTTATCCAGAATCASATCACAGCTWARGGGACTGCCCAGCTGGCAGATGCGTT  
ACAGAGCAACACTGGCATAACAGAGATTTGCCTAAATGGAAACCTGATAAAACCAGAGGA  
GGCCAAAGTCTATGAAGATGAGAAGCGGATTATCTGTTTTCTGAGAGGATGCTTTCTGT  
CATGGGGTTTTTGGCCCTGGAGCCTCAGCAGCAAATGCCACTYTGGGCAGTCTTTTGTGTC  
AGTGTCTTAAAGGGGCCTGCGCAGGCGGGACTATCAGGAGTCCACTGCCTCCATGATGCA  
AGCCAGCTTCCTGTGCAGAAGGTCTGGTTCGGCAAACCTCCCTAAGTACCCGCTACAATTCT  
GCAGAAAAAGAAATGTGTCTTGCGAGCTGTTGTAGTTACAGTAAATACACTGTGAAGAGAC  
TTTATTGCCTATTATAATTATTTTTATCTGAAGCTAGAGGAATAAAGCTGTGAGCAAACA  
GAGGAGGCCAGCCTCACCTCATTCCAACACCTGCCATAGGGACCAACGGGAGCGAGTTGG  
TCACCGCTCTTTTCATTGAAGAGTTGAGGATGTGGCACAAGTTGGTGCCAAGCTTCTTG  
AATAAACGTGTTTGATGGATTAGTATTATACCTGAAATATTTTCTTCCTTCTCAGCACT  
TTCCCATGTATTGATACTGGTCCCACTTCACAGCTGGAGACACCGGAGTATGTGCAGTGT  
GGGATTTGACTCCTCCAAGGTTTTTGTGGAAAGTTAATGTCAAGGAAAGGATGCACCACGG

FIG. 10 (Page 2 of 3)



GCTTTTAATTTTAATCCTGGAGTCTCACTGTCTGCTGGCAAAGATAGAGAATGCCCTCAG  
CTCTTAGCTGGTCTAAGAATGACGATGCCTTCAAAATGCTGCTTCCACTCAGGGCTTCTC  
CTCTGCTAGGCTACCCTCCTCTAGAAGGCTGAGTACCATGGGCTACAGTGTCTGGCCTTG  
GGAAGAAGTGATTCTGTCCCTCCAAAGAAATAGGGCATGGCTTGCCCTGTGGCCCTGGC  
ATCCAAATGGCTGCTTTTGTCTCCCTTACCTCGTGAAGAGGGGAAGTCTCTTCCTGCCTC  
CCAAGCAGCTGAAGGGTGACTAAACGGGCGCCAAGACTCAGGGGATCGGCTGGGAACTGG  
GCCAGCAGAGCATGTTGGACACCCCCCACCATGGTGGGCTTGTGGTGGCTGCTCCATGAG  
GGTGGGGGTGATACTACTAGATCACTTGTCTCTTGCCAGCTCATTTGTTAATAAAATAC  
TGAAAACCCAAAAAAAAAAAAAAAAAAAAAAAAAAGGGCGG (SEQ ID NO:38)

FIG. 10 (Page 3 of 3)

MEEQGHSEMEIIPSESHPHIQLLKSNRELLVTHIRNTQCLVDNLLKNDYFSAEDAEIVCA  
CPTQPDKVRKILDLVQSKGEEVSEFFLYLLQQLADAYVDLRPWLLEIGFSPSLLTQSKVV  
VNTDPVSRYTQQLRHHHLGRDSKFVLCYAKKEELLLEEIYMDTIMELVGFSNESLGSLSL  
ACLLDHTTGILNEQAASRKVTGCVCRTCSSSTTATQSGTPRRCLPSCCASPTWPSSPSMA  
WTSCTRTWT (SEQ ID NO:39)

FIG. 11

CACGCGTCCGCGCTACTGCGGGAGCAGCGTCCTCCCGGGCCACGGCGCTTCCCGGCCCCG  
GCGTCCCCGGACCATGGCGCTCTCCGGGCTCTTCTCTAGCTCTCAGCGGCTGCGAAGTCT  
GTAAACCTGGTGGCCAAGTGATTGTAAGTCAGGAGACTTTCCTTCGGTTTCTGCCTTTGA  
TGGCAAGAGGTGGAGATTGTGGCGGCGATTACAGAAAACATCTGGGAAGACAAGTTGCTG  
TTTTTATGGGAATCGCAGGCTTGGAAGAGACAGAAGCAATTCCAGAAATAAATTGGAAAT  
TGAAGATTTAAACAATGTTGTTTTAAATATTCTAACTTCAAAGAATGATGCCAGAACT  
TAAAAAGGGGCTGCGCAGAGTAGCAGGGGCCCTGGAGGGCGCGGCCTGAATCCTGATTGC  
CCTTCTGCTGAGAGGACACACGCAGCTGAAGATGAATTTGGGAAAAGTAGCCGCTTGCTA  
CTTTAACTATGGAAGAGCAGGGCCACAGTGAGATGGAAATAATCCCATCAGAGTCTCACC  
CCCACATTCAATTACTGAAAAGCAATCGGGAACCTTCTGGTCACTCACATCCGCAATACTC  
AGTGTCTGGTGGACAACCTTGCTGAAGAATGACTACTTCTCGGCCGAAGATGCGGAGATTG  
TGTGTGCCTGCCCCACCCAGCCTGACAAGGTCCGCAAAATTCTGGACCTGGTACAGAGCA  
AGGGCGAGGAGGTGTCCGAGTTCTTCCTCTACTTGCTCCAGCAACTCGCAGATGCCTACG  
TGGACCTCAGGCCTTGGCTGCTGGAGATCGGCTTCTCCCCTTCCCTGCTCACTCAGAGCA  
AAGTCGTGGTCAACACTGACCCAGGTAGGAGTCAGCCCCAGCAAGACCGCAGGCACCAGT  
GCAAGCAGGGGCCCTGGGGGGTTTGGTAATGGCTGGGCCAGCCCTGAGTGCCACCTCAGGA  
AGCAGGCCCAGGTGCTATTTTGATTTTGTAGAAAGGAACAGCTGAATCCTGTCTCCCAAGTG  
CAGCCCAGGTGGCTGCGATTGAACTGCCCCACACCTCGATGGTCTGGTTTATAGAGGGGCC  
TTTGGAAAGTATGGGAATGGCCTGTGTTCTGACCCCTTGCTTTCTTCCTATTCTGACATAT  
GTAGACATTTTAATGGTTGCACAAATTCAAGGTTGTATTTTTTTTTTCTTTAAAAAAATCT  
TTAGCTGGACATGGTAGCACACACCTGTAGTTCCAGCTACTCAGGAGGCTGAGGCAAGAG  
GACTGCTTGAGCCCCAGAGTCTAAGGCTGCAGCGAGCTATGATTGTGCCCCCTACACTCCA  
CAGCCTGGGTTTTAGAGTGAGACCCTGTCTCTAAAAA  
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  
AAAAAAAAAAAAAAAAANGGGCGG (SEQ ID NO:40)

FIG. 12

MEEQGHSEMEIIPSESHPHIQLLKSNRELLVTHIRNTQCLVDNLLKNDYFSAEDAEIVCA  
CPTQPDKVRKIIDLVSQSGEEVSEFFLYLLQQLADAYVDLRPWLLLEIGFSPSLLTQSKVV  
VNTDPGRSQPQQDRRHQCKQGPGGFGNGWASPECHLRKQAQVLF  
(SEQ ID NO:41)

FIG. 13

MEEQGHSEMEIIPSESHPHIQLLKSNRELLVTHIRNTQCL				Majority
10203040				
MEEQGHSEMEIIPSESHPHIQLLKSNRELLVTHIRNTQCL				CARD4-Y CLONE
MEEQGHSEMEIIPSESHPHIQLLKSNRELLVTHIRNTQCL				CARD4-Z CLONE
MEEQGHSEMEIIPSESHPHIQLLKSNRELLVTHIRNTQCL				CARD4L
VDNLLKNDYFSAEDAEIVCACPTQPKVRKILDLVQSKGE				Majority
50607080				
1	VDNLLKNDYFSAEDAEIVCACPTQPKVRKILDLVQSKGE			CARD4-Y CLONE
1	VDNLLKNDYFSAEDAEIVCACPTQPKVRKILDLVQSKGE			CARD4-Z CLONE
1	VDNLLKNDYFSAEDAEIVCACPTQPKVRKILDLVQSKGE			CARD4L
EVSEFFLYLLOQLADAYVDLRPWLLLEIGFSPSLLTQSKVV				Majority
90100110120				
1	EVSEFFLYLLOQLADAYVDLRPWLLLEIGFSPSLLTQSKVV			CARD4-Y CLONE
1	EVSEFFLYLLOQLADAYVDLRPWLLLEIGFSPSLLTQSKVV			CARD4-Z CLONE
1	EVSEFFLYLLOQLADAYVDLRPWLLLEIGFSPSLLTQSKVV			CARD4L
VNTDPVSRYTQQLRHHLGRDSKFVLCYAOKEELLLEEIYM				Majority
130140150160				
21	VNTDPVSRYTQQLRHHLGRDSKFVLCYAOKEELLLEEIYM			CARD4-Y CLONE
21	VNTDPGRSOPQQDRRM-----			CARD4-Z CLONE
21	VNTDPVSRYTQQLRHHLGRDSKFVLCYAOKEELLLEEIYM			CARD4L
DTIMELVGFSNESLGSLSLACLDDHTTGILNEOXXXX--				Majority
170180190200				
51	DTIMELVGFSNESLGSLSLACLDDHTTGILNEOAAASR--			CARD4-Y CLONE
37	-----			CARD4-Z CLONE
51	DTIMELVGFSNESLGSLSLACLDDHTTGILNEOGETIFI			CARD4L
-----XCKKXX				Majority
210220230240				
59	-----KVTG			CARD4-Y CLONE
37	-----QCKQ--			CARD4-Z CLONE
31	LGDAGVGKSMMLLQRLQSLWATGRLDAGVKFFHFRCRMFS			CARD4L
C-----XC-----				Majority
250260270280				
33	C-----VC-----			CARD4-Y CLONE
41	-----			CARD4-Z CLONE
41	CFRESDRLCLODLLFKHYCYPERDPPEEVFAFLLRFPHVAL			CARD4L
-----XXCXXX-----				Majority
290300310320				
36	-----RTCSSS-----			CARD4-Y CLONE
41	-----			CARD4-Z CLONE
51	FTFDGLDELHSDLDLSRVFDSSCPWEPAPHLVLLANLLSG			CARD4L

FIGURE 14 (1 of 4)

----- T X X T X X X X P R ----- Majority			
	330	340	350 360
12	----- T T A T Q S S T P R -----		
41	-----		
21	K E L K G A S K L L T A R T G I E V P R Q F L R K K V L L R G F S P S H L R A Y		
	----- R C Majority		
	370	380	390 400
22	----- R C		
41	-----		
51	A R R M F P E R A L Q D R L L S Q L E A N P N L C S L C S V P L F C W I I F R C		
	----- L P X C ----- Majority		
	410	420	430 440
24	----- L P S C -----		
41	-----		
51	P Q H F R A A F E G S P Q L P D C T M T L T D V F L L V T E V H L N R M Q P S S		
	----- Majority		
	450	460	470 480
28	-----		
41	-----		
51	L V Q R N T R S P V E T L H A G R D T L C S L G Q V A H R G M E K S L F V F T Q		
	----- G P G G ----- Majority		
	490	500	510 520
28	-----		
41	----- G P G G -----		
51	E E V Q A S G L Q E R D M Q L G F L R A L P E L G P G G D Q Q S Y E F F H L T L		
	----- P X X X W X X P ----- C X X Majority		
	530	540	550 560
28	----- C A S		
45	----- F G N G W A S P -----		
21	C A F F T A F F L V L D D R V G T Q E L L R F F Q E W M P P A G A A T T S C Y P		
	----- P ----- Majority		
	570	580	590 600
41	P		
53	-----		
51	P F L P F Q C L Q G S G P A R E D L F K N K D H F Q F T N L F L C G L L S K A K		
	----- X W X ----- Majority		
	610	620	630 640
42	----- T W P -----		
43	-----		
51	K E L L R H L V P A A A L E R K E K A L W A H L F S S L R G Y L K S L P R V Q V		

FIGURE 14 (2 of 4)

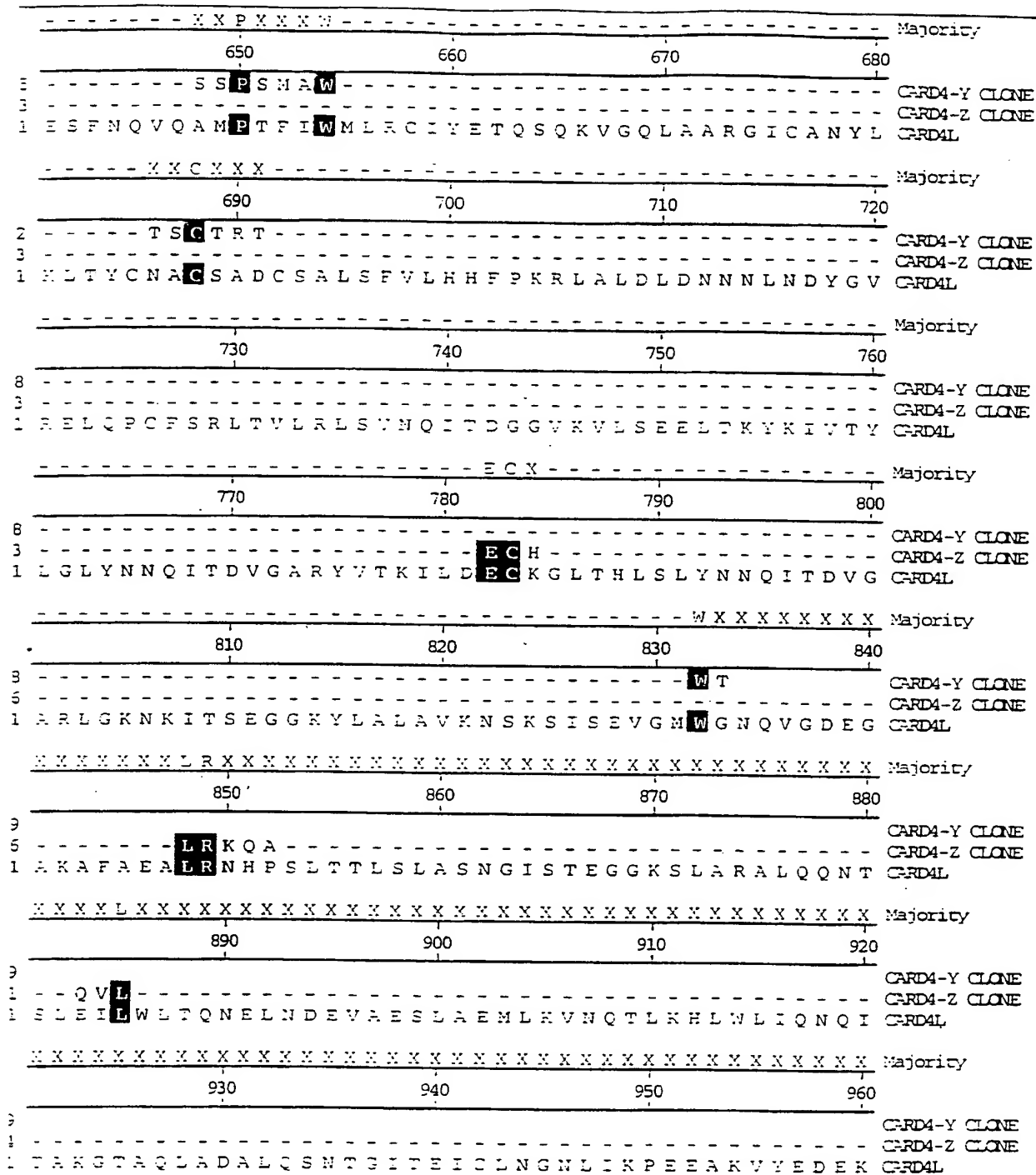


FIGURE 14 (3 of 4)

<u>XXXXF</u>		Majority
19		
64	- - - - F	CARD4-Y CLONE
61	R - - - C F	CARD4-Z CLONE
		CARD4L
decoration (Decoration #1): Shade (with solid black) residues that match the Consensus XACTIV.		

FIGURE 14 (4 of 4)



CCACGCGTCCGCGGACCGCGAGCGGTAGCGCCCTCCCTCCCAGCTGTTGTCCCGCCCGAT  
CCGCGACCCTAGTCCCCGGATCCCCTTGCTGAGAGTCACCGTACTCCAGGGCCAACTGAG  
CCAAAGTCCTGCCAACTTGGGTGAGCAATGAAAGGCAGGATCCTGGGTGGTGGCCCTGAA  
TCCTGATTTGTCTGCCCTGCCAGCGAGACACATGTGGTCAAAGATGAATTTGAGAAAAGT  
AGCTGCTGGCTACTTGAACAATGGAGGAACACGGCCATCATGAGATGGAAGGCACCCCAT  
TGGGTTGTCACTCCCACATTAAACTGCTGAAGATCAACAGGGGAACATCTGGTCACCAACA  
TTCGGAACACTCAGTGTCTGGTGGACAACCTTGCTGGAGAATGGCTACTTCTCAGCCGAAG  
ATGCAGAGATTGTGTGTGCCTGTCCCACCAAGCCTGACAAGGTCCGAAAGATCCTTGACC  
TGGTGCAGAGCAAAGGCGAGGAGGTGTCTGAGTTCTTCCTCTACGTGCTGCAGCAGCTGG  
AGGATGCTTACGTGGACCTCAGGCTGTGGCTCTCAGAAATTGGCTTCTCCCCTTCCCAGC  
TCATTCCGACCAAACTATCGTCAATACTGACCCAGTAAGCAGGTATACCCAACAGCTGC  
GACACCAACTGGGCCGCGACTCCAAGTTCATGCTGTGCTACGCCCAGAAGGAGGACCTGC  
TGCTGGAGGAGACCTATATGGACACACTCATGGGGCTGGTAGGCTTCAACAATGAAAACC  
TGGGCAGCCTAGGAGGCCTGGATTGCCTGCTGGACCACAGTACGGGCGTCTCAACGAGC  
ATGGCGAGACTGTCTTCGTGTTCCGGGACGCGGGAGTGGGCAAGTCCATGCTGCTGCAGA  
GGTTGCAGAGCCTCTGGGCGTCAGGCAGGTTGACCTCCACAGCCAAATTCTTCTTCCACT  
TCCGCTGCCGCATGTTTCAGCTGCTTCAAGGAGAGCGACATGCTGAGTCTGCAGGACCTGC  
TCTTCAAGCATTTCTGCTACCCGGAGCAGGACCCCGAGGAGGTGTTCTCCTTCTTGCTGC  
GCTTTCCCCACACAGCGCTCTTCACTTTTGACGGCCTGGATGAGCTGCACTCAGACTTCG  
ACCTGAGCCGCGTGCCGGATAGCTGCTGCCCCCTGGGAGCCGGCTCACCTCTGGTCCTGC  
TGGCTAACCTCCTAAGTGGGAGGCTGCTCAAGGGTGCCGGCAAATTGCTCACTGCTCGCA  
CAGGCGTGAGGTCCCCCGCCAGCTCCTGCGCAAAAAGGTGCTGCTCCGGGGCTTCTCCC  
CAAGTCACCTGCGCGCCTATGCCCGCCGGATGTTCCCCGAGCGCACAGCGCAGGAGCATC  
TGCTGCAGCAGCTGGATGCCAACCCCAACCTCTGCAGCCTGTGCGGGGTGCCGCTCTTCT  
GTTGGATCATCTTCCGTTGTTTCAGCACTTCCAGACGGTCTTCGAGGGCTCCTCTTCAC  
AGTTGCCGACTGTGCTGTGACCCTGACCGATGTCTTCTGCTGGTCACTGAGGTGCATC  
TGAACAGGCCGCGAGCCAGCAGCCTGGTGCAGCGCAACACGCGCAGCCCCGGCGGAAACCC  
TACGTGCAGGCTGGCGCACGCTGCATGCGCTGGGAGAGGTGGCTCACCGAGGCACCGACA  
AGAGCCTCTTTGTGTTTGGCCAGGAGGAGGTGCAGGCGTCGAAGCTGCAGGAAGGAGATC  
TGCAGCTGGGCTTCTGCGGGCTTTGCCCGATGTGGGGCCCTGAGCAGGGCCAGTCTTACG  
AATTTTCCACCTTACGCTCCAGGCCTTCTTCACCGCCTTCTTCCTGGTAGCAGATGACA  
AAGTGAGCACCCGGGAGTTGCTGAGGTTCTTTCGAGAATGGACGTCTCCTGGAGAGGCAA

FIG. 15 (Page 1 of 3)

CAAGCTCGTCCTGCCATTCTTCCTTCTTCTCCTTCCAGTGCCTGGGCGGCAGAAGCCGGT  
TGGGCCCTGATCCTTTTCAGGAACAAAGATCACTTCCAGTTCACCAACCTCTTCGTGTGCG  
GGCTACTGGCCAAAGCCCCGACAGAACTCCTTCGGCAGCTGGTGCCCAAGGCTATCCTGA  
GGAGGAAGCGCAAGGCCCTGTGGGCTCACCTGTTTGCTAGCCTGCGCTCCTACTTGAAGA  
GCCTACCTCGGGTCCAGTCTGGAGGCTTTAACCAGGTGCATGCCATGCCACATTCCTGT  
GGATGCTGCGCTGCATCTATGAGACGCAGAGCCAGAAGGTGGGGCGCCTCGCCGCCAGGG  
GCATCAGTGCGGACTACCTCAAGCTGGCCTTTTGCAACGCTTGCTCTGCGGACTGCAGCG  
CCCTGTCTTCGTCTGTCATCACTTCCACAGGCAGCTGGCCCTAGACCTGGACAACAACA  
ACCTCAATGACTATGGCGTGCAGGAGCTGCAGCCTTGCTTTAGCCGTCTCACGGTTATCA  
GACTCAGCGTCAACCAGATCACCGACACGGGGGTGAAGGTGCTATGTGAGGAAGTACCA  
AGTATAAGATCGTGACGTTCTTGGGTTTATACAACAACCAGATAACTGATATCGGAGCCA  
GGTATGTGGCCCAAATCCTGGATGAATGCAGAGGCCTCAAGCACCTTAACTAGGGAAAA  
ACAGAATAACAAGTGAGGGCGGGAAGTGTGTGGCTTTGGCTGTGAAGAACAGCACCTCCA  
TCGTTGATGTTGGGATGTGGGGTAATCAGATTGGAGACGAAGGGGCAAAGGCCTTCGCAG  
AGGCATTGAAGGACCACCCAGCCTGACCACTCTCAGTCTTGCAATCAATGGCATCTCTC  
CGGAGGGAGGGAAGAGCCTTGCGCAGGCCCTGAAGCAGAACACCACACTGACAGTAATCT  
GGCTGACCAAAAATGAACTTAATGATGAGTCTGCAGAGTGCTTCGCTGAGATGCTGAGAG  
TGAACCAGACGCTACGGCATTATGGCTGATCCAGAATCGCATCACAGCCAAGGGGACAG  
CGCAGCTGGCGAGGGCACTGCAGAAGAACACAGCCATAACAGAGATTTGTCTCAATGGAA  
ACTTGATTAAGCCCGAGGAGGCCAAAGTCTTCGAGAATGAGAAGAGAATCATCTGCTTCT  
GACGGACGCTCCTGGGCAGGATCTTTGTCCTAGGTTGCTCCTCAGTCACAGACAGCACTG  
TGCAGTCAGCAGGGTAGCAGGATGCTGTGCAGCGCCTGCAGCAAGGTGCCTGTCAGGAGC  
CCACACCTCCACAGTGCACACCGATGTCCCCTGCTCATGCTTGGAAGTGGTAGCACCCGCG  
CCGCGGCTGAGACCCTGCAGACGCAGGGAGTCTTAGGAACCATCGTCACCACTCAAAGCC  
AGCAGGGCATCTTCTGTACAAAGATCTCCCTGCATATCCACTAGACGGAAGCTGAAGGAA  
CGCAACAGCAGAGGAGGCCAACAGACGCCTGGCTGAAGGCTCCGTGGGACCAACGGTGTC  
ACCTTCAGAAAAGAGCTGGGAACTTGAGCAGAGCCGATGGTAACTTCTTGGGGAAAGAAG  
GCACCCAGTGAAGTGCATGGTTATTCTGAGTCTCCTTCCTCTGCTTAGTCCCTCTCACTG  
TACAGGTCTGTTTCTTCCTCGCAGCTGTGGCTGCTGAAGTAGGTCCACTGTGGGGAGAGC  
TCATCACAGACTTTGGTTTCGGTTCTGGATTCTCAGTGGTGGCAACCGAGAGTCAGACGAT  
ACCCTCTAGGTCACTCTCAGAGGATCTCTATGCTGTGAGAGGGTTGAGGGCCCCACCCAGA  
ATTTTTTTTTTTTACCAGTTTTTACTGTGCCTGCCCCAGGAGGGAGAATTACTTCCCAGC

FIG. 15 (Page 2 of 3)

CTCCACAGCAGCAGGCATGGCTTGCCTCAATGGTCCTGAGATCCCAACAAACTCTCTCC  
CTTGCCTGTGAGCAGAAAGTATCTTCATGTCCTCAGAAGTTGGAGGGTGACTGGACACAG  
TTAAGACTCAGAGAGCCAGCTGATAGCTCAAAGCAAAGCATGGCACATACCCACCACCAT  
ACCATGGTGCGCATGGGATGGGACAGTTGGAATGTTGCAGATAACGTGTTCTTTTGCCAG  
TTCATTTGTTAATAAAATATTTAAAACGTTAAAAAAAAAAAAAAAAAAAAAAAAAAGGGCG  
G (SEQ ID NO:42)

FIG. 15 (Page 3 of 3)

MEEHGHHEMEGTPLGCHSHIKLLKINREHLVTNIRNTQCLVDNLLENG YFSAEDAEIVCA  
CPTKPKDKVRKILDLVQSKGEEVSEFFLYVLQQLLEDAYVDLRLWLSEIGFSPSQLIRTKTI  
VNTDPVSRYTQQLRHQLGRDSKFMLCYAQKEDLLEET YMDTLMGLVGFNNENLGS LGGL  
DCLLDHSTGVLNEHGETVFVFGDAGVGKSM LLQRLQSLWASGRLTSTAKFFFHFRCRMFS  
CFKESDMLSLQDLLFKHFCYPEQDP EEVFSFLLRFPHTALFTFDGLDELHSDFDLSRVPD  
SCCPWEPAHPLVLLANLLSGRLLKGAGKLLTARTGVEVPRQLLRKKVLLRGFSPSHLRAY  
ARRMFPERTAQEHLLQQLDANPNLCSLCGVPLFCWII FRCFQHFQTVFEGSSSQLPDCAV  
TLTDVFLLVTEVHLNRPQPSSLVQRNTRSPAETLRAGWRTLHALGEVAHRGTDKSLFVFG  
QEEVQASKLQEGDLQLGFLRALPDVGPEQGQSYEFFH LTLQAFFTAFFLVADDKVSTREL  
LRFFREWTSPGEATSSSCHSSFFSFQCLGGRSRLGPD PFRNKDHFQFTNLFVCGLLAKAR  
QKLLRQLVPKAILRRKRKALWAHLFASLSYLKSLPRVQSGGFNQVHAMPTFLWMLRCIY  
ETQSQKVGRLAARGISADY LKLAFCNACSA DC SALS FVLH H FHRQLALDLDNNNLNDYGV  
QELQPCFSRLTVIRLSVNQITDTGVKVLCEELTKYKI VTFGLYNNQITDIGARYVAQIL  
DECRGLKHLKLGNRITSEGGKCV ALAVKNSTSI VDVGMWGNQIGDEGAKAF AEAL KDHP  
SLTTLSLAFNGISPEGGKSLAQALKQNTTTLTVIWLTKNELNDESAECFAEMLRVNQTLRH  
LWLIQN RITAKGTAQLARALQKNTAITEICLNGNLIKPEEAKVFENEKRIICF  
(SEQ ID NO:43)

FIG. 16

25 / 35

**FIG. 18 (1 of 10)**

tgtatgattctgtttatatgaaatgtccagaaaaggtaaattctatagacaaagcaaatacagtagt  
tgcctacggccagggttggctacaaataggctccagaaaactctgggaagatggtagagatgt  
tctagacctggactgtgggtgaggtttgcacaactttgtaaacttactaaaaattactgacaaata  
tataacactccctaacactttgggaggccgaggtgggcagatcgcttgaacccaggaatttgaga  
ccagcctgggcaacatggcgagaccccgctctacaaaaaacacaaaaattagttgggcttgggt  
ggcatatgacctgtgtcccagctacttgggaggtgaggtgggaggattgcttgagcctgggagtt  
tgagactgcatgattgggtcactgcaccctagcctgagtgcagagcaggaccctatctctaaca  
acaaaaaagcagtggttgggtggaggaggccagcgtggccatctggcctggccctcgagtgcgagg  
ggcttcagtggttagctgcagttcagtgatgacactgtgcggaggaataagggtggcctgtctca  
gacactgatcccagctgaagtttgtcaccttctttctggcaaatctgaggtcaagcagagagatc  
aaagcctggggccctcagggtcaggaatgctggctctgtgacgctccccagggtcctgcatctgag  
gagtggtgctgcgctggcctcagggccagggttgtaattttgtttatgcaactcgccctctcctcttt  
gagacctcctgtttgatgctgtttctgcctctctcctcaccctgctgctgtgacctgccacccc  
ctccctccagtgagcaggtatacccagcagctgcgacaccatctgggcccgtgactccaagtctgt  
gctgtgctatgcccagaaggaggagctgctgctggaggagatctacatggacaccatcatggagc  
tggttggcttcagcaatgagagcctgggcagcctgaacagcctggcctgcctcctggaccacacc  
accggcatcctcaatgagcaggtgagaccatcttcatcctgggtgatgctggggtgggcaagtc  
catgctgctacagcggctgcagagcctctgggcccacgggcccggctagacgcaggggtcaaattct  
tcttccactttcgctgcgcgatgttcagctgcttcaaggaaagtgcagagctgtgtctgcaggac  
ctgctcttcaagcactactgctacccagagcgggacccccaggaggtgtttgccttccctgctgcg  
cttccccacgtggccctcttccacttcgatggcctggacgagctgcactcggacttggacctga  
gccgctgctgacagctcctgccccctgggagcctgccacccccctgggtcttgcctggcacaactg  
ctcagtgggaaagctgctcaaggggctagcaagctgctcacagcccgcacaggcatcgaggtccc  
gcgcaggttcttgcggaagaagggtgcttctccggggcttctccccagccacctgcgcgcctatg  
ccaggaggatgttccccgagcgggcccctgcaggaccgctgctgagccagctggaggccaaacccc  
aacctctgcagcctgtgctctgtgccccctcttctgctggatcatcttccgggtgcttccagcactt  
ccgtgctgccttgaaggctcaccacagctgcccagctgcacgatgacctgacagatgtcttcc  
tccctggctcactgaggtccatctgaacaggatgcagcccagcagcctgggtgcagcggaaacacagc  
agcccagtgaggagacctccacgcccggccgggacactctgtgctcgctggggcaggtggcccaccg  
gggcatggagaagagcctcttgtcttcacccaggaggaggtgcaggcctccgggctgcaggaga  
gagacatgcagctgggcttccctgcgggcttggccggagctgggccccgggggtgaccagcagtc  
tatgagttttccacctcaccctccaggccttctttacagccttcttccctcgctgctggacgacag  
ggtgggactcaggagctgctcaggttcttccaggagtggatgccccctgcgggggagcgcacca  
cgctctgctatcctcccttccctcccggtccagtgccctgcagggcagtggtccggcgcggaagac  
ctcttcaagaacaaggatcacttccagttcaccaacctcttccctgtgcgggctgttgtccaaagc  
caaacagaaaactccttcggcatctggtgcccgcgggcagccctgaggagaaagcgcaaggccctgt  
gggcacacctgttttccagcctgcggggctacctgaagagcctgccccgcgttcaggctcgaaagc  
ttcaaccagggtgcaggccatgcccacgttcatctggatgctgcgctgcatctacgagacacagag  
ccagaagggtggggcagctggcgccaggggcatctgcgccaactacctcaagctgacctactgca  
acgcctgctcggccgactgcagcgccctctccttcgtcctgcatcacttccccaaagcgggctggcc  
ctagacctagacaacaacaatctcaacgactacggcgtgcgggagctgcagccctgcttcagccg  
cctcactgttctcaggtgaggctgccaggcaaggggagcaacaggtgggcccgggcccgggct  
cggaggggcatcggaatggcatcatggaccaggatccccaggactcatgacctggcccttgga  
atgtccagaccttttcttcttagcagggcagaggtcaaggtgcaaagcttcgaggcaggtggac  
ctggatcagccacagctgggtgcccctgaacaaagtgttaactctcagagcctccacgcccctca  
tctggaaaaagaagatgctcataatcctatcaattatggccacagggaccaatgttagttgagaa  
tgggtgaagtgcattacaaatattacctaatggaatgctctttacaacctgttaacttaggtact  
gttattgtctctatttttggcagataaggaagttagaggcacagagaagtttaagcttgccttagg  
tcacacagctcagacatagcagtgccagaatgcataaagaaccttcccttttaagattaatgtaag  
gctccgagatagccctcaaaaagtttctggaatatgggagcttttattactgcagagaaagcaga  
ccttgtgccagttggcactgggtgactttctgtgatcaacgctagcagcccttcacactgctagag  
acctcagttaaaatgctgactcgtgggtgttttctgttccatagtttacgggaaacagagccca  
gtctgttttcttctattagcatttccctatgtaaaataaaccttgtaaatctctacagggggttaa  
atttgcattacttgactcatgcatttctaaaaagcagtagggatttggaactgactcccagtg

FIG. 18 (2 of 10)

ctgtcacaccagtgtcagagtgtataaataattgcatggggacatgggggtgcaggggggtcgaaggct  
gccctagcctgggaattggaaaacctggagtctgttctctgtactctcagccagtgtactctccct  
ctgtagccccaggcaggtctcacactcagtgccacctctgtccatcttttttttctccccaa  
atggagtcccgtctgttggccaggctggagtgcagtgccgtgatctcagctcactgcaacctcc  
gcctcctgggttcaagcgattctcttgccccagcctcctgagtagctgggattacaggcacacgc  
caccatgtccggctaagttttttgtatttttagtaggacgggggtttcccatgttggccaggctg  
gtcttgaaatcctgacctcaggtgatccgcccgcctcggccttccaaaatgttgggggttacaggc  
atgagccgcgcacccgacccctctgtccatcttttcaatgggaaactccacaccagtgtggtgg  
cctgccccttctgtctgtccccagggtgaagctttccttcacaccagtgcagaaaaaacagcttg  
taggaaagcagaggatagggtaaccacgggaagcacactcagttctctggctgcatcagttagg  
attagtttttagctgagagcgaaaaccccaaatgttgggtgagttacaagcttatttctctcatgta  
aaagtctagaggtaggtagttcaggactgggtatggagtctccatgacctccggagcccaggctc  
tcttctgccttctgttctgccatcctcactaccggctttcccatcttgcccagaggggtgc  
tcaactccagccatctagtgcacactctagctatcagtaagaaggaagggcaaagattgagagc  
atgcctcaatcttttaagaacacttcttgggtattactaattatattgctgcttagatttcagaa  
cttaatgggtatgggcagaatttaatgagatgggcccagctaaaagatgggggaatctattgctaa  
gaaagtatagatattgggaatgtctagcagcctgtgctgtcttgggctggccatgccatgtacat  
acacactatttcccagcaccaagctggggactctgagggaaaggggtccagagtgtctgacttgat  
cattttgatgtggcctaaaaatcaagcttttaattgttcagccttttacttggtatcaaggtcag  
cttgtgggtctaattgggcccaggcttgtgtttctaaagtaagttttatttggaaacgcagccata  
cccatttatttacttactggctgttccactacacagttgagttagctgtgacagagaccacatg  
gccacagagcctaaaaatatttgtgtctgacactttacagaatgacatgagcagttcctttga  
cagtgggactcacagccttttccagtgcacaaatcagggttagcccatgtgtttctggatgggggg  
aagctgttggcattttgggtataacagttcttgtgagacctgtccagcattttgcaggacaccta  
acatcattggccctgcctgcaagatgacagggcactccctcctccagtcacaaccactaaaagca  
gccctgacatttccaaacccatgccctccaccatacagagaaccagggtacagggtctggctgaca  
cataggtcacacgcaaaggggtggatgtcagaggtggctggcctcacacgtcctccctgtgtcctt  
cacggctcgtgtgaggagccaggggctgtgtgcagcctcgctcatgggctgggtgcaggatgggtc  
tggcgccccacgttggccaggctttgtaaagggctatttggctgattgctgtggcattctcca  
ggggcgctctatacctgagaaaactccagggtcgaaaggttctggatctttgtaagattaatgggt  
ccttcataatgagtgccctgacctgactcgtaattttttgtgtttttatttcagactcagcgtaa  
accagatcactgacgggtggggtaaggtgttaagcgaagagctgaccaaatacaaaattgtgacc  
tatttgggggtatgtctttctccagaacactgggccaactacctagtaataatacagagctgcagg  
gaattcacattcccataggtccctggatgatcggcacggatggcccaggggtgggaagagcgctg  
gcccaggagttgagagtccctgggttctcttgtggctcggccagtcaggaagtcttgcagcct  
cagcctcctcacctgtaaaactgggatcccagtataggcaagtaggcttacaactgggtattggg  
ggatgcaacgagaatataaaggggatataatttaataaatgctagaatcctgtttacatattagtct  
ggactattttgggtccataatccctcatccagagcctttggggcaagaccgaatggggattctg  
agtgcagctatggcatgacgtggccgcagggtctaaggcagtgccccattttcaaacactttc  
atatttctcccgcagaatgtatgaaacagtgaaaccaaggttggttaagaaagactataagtagct  
ccacatcagttgccaaaagaattgtgagaaactttgggcattcagagcctttgaggttttggagt  
ctgagagaagggattgcggggccagccccacacaactgggtggctctgcaagctggagcagttgttc  
agtttcttggggcctcagtgcccttcgatgttaatgaggacatggacgcaaacgaccccgggcca  
cactcggtccagggtctgtgtggctgtggaaccctggaagcctgagcttagctgcctttcaac  
ttccatctgtgtactattgaattggcattgagcgggtgagatggctgaaaggttagacatcgagaa  
gttttaatatcagaatcttttcttcaagacgctgaatgtaatcttagttgtaaatacccatc  
acctgccagtcccgagcactcatgcaccagggtttgcgttatgtcctaagatcctcataacca  
ccctgcaaggggactatcatcattacctctgtattacagatggagaaactgaggcacagagaggt  
aacgtgacttgtctcaggccataaagctggggaaagttagtgagctgggttttgaacctgagctgt  
gagacctcagagccctaaactctggtcctgtgttccccctttcaaccagactttgggaatca  
gtagaccacatagcttcaaaaaaacaggggctattaaaatgacatcaggagccagaaagtctcat  
ggctgtgtcttctcttgaagtttatacaacaaccagatcaccgatgtcggagccagggtacgtcac  
caaatcctggatgaatgcaaaggcctcacgcattcttaagtaagtggggtaggcaccagggtcct  
tagtatattctcttgatcaccccttctgttgttcaaagattaaatgtcacagtaagagagcttctc

FIG. 18 (3 of 10)



atcctaaagccttccacttgtcccagggccatgttgggtcaagtaaaagatacctctgtgtgatctg  
tgaggcttggattctggaagggcctcccgttatttggtagggggaaagggttggcatttttgatttca  
ttaactactaggccgaagaaaggactaactctcaccctttctggtggtctttttgccccaaaggga  
gtttcctgtcgggttgcaaggaagagcttgggccccttgccctgctgtaggtgtgcccctgcgagg  
gggtgacagtgcgccaggcttggagcctctggtcctgcccctgacagtgggccacataccttgaccc  
ttggcagtcacaagtgggaccccccagggtctcccagggaagtcaagtgtgctgctgaggtcaatt  
agaggaccccaggagggtcaggtccctgagcttctgcagagactgtggaccatctcctggaga  
ggaaccctgactgactgtcctcagggcttcagttccctccctgacaggaggcccaggccatgggt  
cttgtggatcccagaagaaagtgtacggttcccaagatggggctggaaggggctctgtgctgggg  
aggaggggtgaccacattggagcccctgcatagctggaggctgactgtgtgtgactctctctgca  
gactgggaaaaaacaataaacaagtgaaggagggaagtatctcgccctggctgtgaagaacagc  
aatcaatctctgaggttgggtgagtagaaggggatggatgtatgtggtacaacctgctgtgtgt  
gtggggggcgggccttgctgttcttttcatacatcagtagaccagaaggaccactggggctcgct  
gtcggggagagatagtgagagcttccaccatgctgcgaaactgaaaccgtgccattaaagcaat  
aactccccggtccccctccccctgccccttgcagcccaccctgctacttactctctctatggttt  
tgactactctacctcatgtaagtgaatcatacagtatattgccttttggggatggctgatttcac  
tagcatcatgtcctcaagattcgtccacatggaagcatgggacaggatttcctttttttttttt  
tttttttttttttgacagagctctcgctctgttgcccaggctggagtgacagtgccatgatctcgg  
ctcactgcaacctctgccttctgggttcaagcgattctctcgccctcagccacacgagtagctggg  
attataggcacccggccaccaatcccagctaatttttgtatttttagtagaggcggggtttcacca  
tgttggccaggctggtctcaaaactcctgacctcaaatgatccaccacacctcgggtctcccaaagt  
tcaggattataggcgtgagccaccgtgccccggcaggatttccttctttttaaggctgagtaat  
actccattgcatggctatgccacattttgtttactcattcatccaagaacagacactggcttgc  
tctatgctttggctgttgtgaataatgctgctgtgcacatggggcatacaaatgtctctcaagg  
ctgccttcaattcttttttttttttttttttttttagattctttttttttttttattatactcta  
agtttttaggtacatgtgcacattgtgcaggttagttacatatgtatacatgtgccatgctgggtg  
cgctgcaccactaatgtgtcatctagcattaggtatatctcccaatgctatccctccccctcc  
ccgacccccaccacagtcccagagtgatattccccttccctgtgtccatgtgatctcattggt  
caattcccacctatgagtgagaatatgcgggtgtttgggttttttgttcttgcgatagtttactgag  
aatgatgggttccaatttcatccatgtccctacaaaggatagaactcatcatttttatggctg  
catagattcccatgggtgtatatgtgccacatttcttaattcagctcatcattgttggacatttg  
gggtgggttccaagtcttgcatttgtgaatatgtgcccacataaacatacgtgtgcatgtgtctt  
atagcagcatgatttatactcatttgggtatataccagtaaatgggatgggtgggtcaaagtga  
tttctagttctagatccctgaggaatcgccacactgacttccacaatgggtgaactagtttacag  
tcccaccaacagtgtaaaagtgttccatttctccgcacccctctccagcacctgctgttccctga  
ctttttaatgattgccattctaactgggtgtgagatgatctcatagtgggtttgatttgcattt  
ctctgatggccagtgtgatgagcatttcttcatgtgttttttggctgcataaatgtcttcttt  
gagaagtgtctgttcatgtccttgcgccactttttgatgggggttgttgttttttcttgtaaat  
ttgtttgagttcattgtagattctggatattagcccttgtcagatgagtaggttgcgaaaattt  
tctcccatgttgtaggttgcctgttcaactctgtatggtagtttcttttgcctgtgcagaagctctt  
agtttaattagatccatttgtcaatttgtcttgggttggcatgtcttgggtgttttgacat  
gaagtcccttgcgccacgcctatgtcctgaatggtaatgcctagggtttcttctagggttttatgg  
tttttaggtttaacgtttaaatctttaatccatcttgaattgatttttgtataagggtgaaggaag  
ggatccagtttcagctttctacatatggctagccagttttcccagcaccatttattaaataggga  
atcctttcccatgtgttgttttctcaggttgtcaaagatcagatagttgtagatatgcggca  
ttatttctgagggctctgttctgttccattgatctatatctctgttttgggtaccagtaccatgct  
gttttgggttactgtagcctttagtatagtttgaagtcaggtagtgatgcctccagctttgtt  
cttttggcttaggattgacttggcgatgcgggctcttttttgggtccatatgaactttaaagtag  
tttttccaattctgtgaagaaagtcattggtagcttgatggggatggcattgaatctgtaaat  
accttgggcagtatggccatttccacgatattgattcttccctacccatgagcatggaatgttctt  
ccatttgtttgtgtcctcttttatttcccttgagcagtggtttgtagttctccttgaagaggtcct  
tcacatcccttgaagttggattccttaggtattttattctcttgaagcaattgtgaaggggagt  
tcacccatgatttggctctctgttgtgtgttgggtgtataagaatgcttgtgatttttgtac  
attgattttgtatccctgagacttgcctgaagttgcttatcagcttaaggagattttgggctgaga

FIG. 18 (4 of 10)

cgatgggggttttctagataaacaatcatgtcgtctgcaaacagggacaatttgacttcctctttt  
cctaattgaataccctttatttcttctcctgcctgattgccctggccagaacttccaacactat  
gttgaataggagcggtagagagagggcatccctgtcttgtgccagttttcaaagggaatgcttcca  
gtttttgcccattcagtatgatattggctgtgggtttgtcatagatagctcttatttttgaaa  
tacgtcccatcaatacctaattttattgagagtttttagcatgaagggttgttgaattttgtcaaa  
ggctttttctgcatctattgagataatcatgtgggtttttgtctttggctctgtttatatgctgga  
ttacattttattgattttgcgtatattgaaccagccttgcàtcccagggatgaagcccacttgatca  
tggttgataagctttttgatgtgctgctggatttcgggtttgccagttttttattgaggatttttgc  
atcaatgttcatcaaggatattggctctaaaattctcttttttggttgtgtctctgcccggctttg  
gtatcagaatgatgctggcctcataaaatgagttagggaggattccctctttttctattgattgg  
aatagtttcagaagggaatggtagcagttcctccttgtacctctggtagaatttcggctgtgaatcc  
atctggctcctggactcttttttggttggttaaactattgattattgccacaatttcagagcctgtta  
ttggtctatttcagagattcaacttcttctcctgggttagtcttgggagagtgatgtgtcgaggaat  
gtatccatttcttctagattttctagtttatttgcgtagagggtgtttgttagtattctctgatgg  
agtgtgtatttctgtgggatcgggtggatgatccctcttcatctttttattgtgtctattttgat  
tcttctctcttttttcttattagttctgtctagcgggtctatcaattttgttgatcctttcgaaa  
aacagctcctggattcattgattttttgaagggtttttgtgtctctatttcttccagttctgc  
tctgatttttagttatttcttgccttctgctagcttttgaatgtgtttgtctcttcttctagtt  
cttttaattgtgatgttaggggtgtcaattttggatctttcctgcttctcttgtaggcatttagt  
gctataaatttccctctacacactgctttgaatgcgtcccagagattctgggtatgtgggtgtctt  
gttctcggttggttcaaagaacatctttatttctgccttcatttcggtatgtaccagtagtcat  
tcaggagcaggtgttccagtttccatgtagttgagcggctttgagtgaagattcttaatcctgagt  
tctagtttgattgcactgtggctctgagagatagtttgttataatttctgttcttttacatttgc  
gaggagagctttacttccaactatgtgggtcaattttggaataggtgtgggtgtggtgaaa  
atgtatattctgttgatttgggtggagagttctgtagatgtctattaggtctgcttggtgcaga  
gctgagttcaattcctgggtatccttgttgacttctgtctcattgatctgtctaatgttgacag  
tgggggtgttaaagtctcccatatttaattgtgtgggagtcctaatctcttgttaggtcactgagga  
cttgccttatgaatctgggtgctcctgtattgggtgcataaatatttaggatagttagctcctct  
tgttgaattgatccctttaccattatgtaatggccttctttgtctcttttgatctttgttggttt  
aaagtctgttttatcagagactaggattgcaacccctgcctttttttgttttccattggcttggt  
agatcttctcccatcctttttatttttgagcctatgtgtgtctctgcacgtgagatgggtttcctga  
atacagcacactgatgggtcttgactctttatccaacttgccagtcctgtgtcttttaattgcaga  
atttagtccatttatatttaaagttaatatgtttagtgtgaatttgatcctgtcattatgatgt  
tagctggcgatttgcctcattagttgatgcagtttcttctcctagtcctcgatggcttttacattttg  
gcatgattttgcagcggctggtagccggtgttcccttccatgtttaccgcttcttccaggagctc  
ttttagggcaggcctgggtggtagcaaaatctctcagcatttgcttgtctataaagtattttattt  
ctccttcacttatgaagcttagtttggctggatataaaattctgggttgaaaattcttttcttta  
agaatgttgaatattggccccactctctctggttgttaggggtttctgcccagagagatccgctgt  
tagtctgatgggctttcctttgagggtaacccgaacttctctctggtgccccttaacatttttt  
ccttcattttcaactttgggtgaatctgacaattatgtgtcttggagttgctctctcgaggagat  
ctcctggataaatatcctgcagagtggtttccaacttggttccattctccacatcactttcaggta  
caccaatcagacgtagatttgggtcttttcacatagtcacatatttcttggaggctttgctcattt  
ctttttattcttttttctctaaacttcccttctcgcttcatttcatttcatttcatttccattgc  
tgataccctttcttccagttgatcgcatcggtcctgaggcttctgcattcttcacgtagttctc  
gagccttggttttcagctccatcagctcctttaagcacttctctgtattgggtatttctagttata  
cattcttctaaatttttttcaaagttttcaacttctttgcctttgggtttgaatgtcctcccgtag  
ctcagagtaatttgatcgctgaagccttcttctctcagctcgtcaaaatcatttctccatccagc  
tttgttctgttgcgtggtaggaactgcgttccctttggaggaggagaggcgtctgcgttttagag  
tttccagtttttctgttctgttttttccccatctttgtgggtttatctacttttgggtctttagatg  
atggtgatgtacagatgggttttcagtgtagatgtcctttctgggtgttagttttcttcttaaca  
gacaggaccctcagctgcaggtctgttgggaataccctgcccgtgtgaggtgtcagtggtgcctctgc  
tggggggtgcctccagttaggctgtcgggggtcaggggtcagggacccttgaggaggcaggt  
ctgcccgttctcagatctccagctgcgtgctgggagaaccactgctctcttcaaagctgtcagac

FIG. 18 (5 of 10)

agggacacttaagtctgcagagggtactgctgtctttttgtttgtctgtgacctgccccagagg  
tggagcctacagaggcaggcaggcctccttgagctgtggtgggctccaccaggttcgagctccc  
ggctgctttgtttacctaagcaagcctgggctatggcgggcgccccctccccagcctcgttgccg  
ccttgacagtttgatctcagactgctgtgctagcaatcagcgagattccgtgggcgtaggaccctc  
tgagccagggtgtgggatatagtcctgtggtgcgcctgttcttaagccgggtctgaaaagcgcaata  
ttcgggtgggagtgaccgattttccagggtgcgtccgtcacccttctttgactcggaaaggga  
actccctgatcccttgcgcttcccagggtaggcaatgcctcgccctgcttcggctcgcgcacggg  
gcgcgcacacactggcctgcgcccactgtctggcgctccctagttagatgaaccgggtacctcag  
atggaaatgcagaaatcaccctgtcttctgcgtcgtcacgctgggagctgtagaccggagctgtt  
cctattcggccatcttggtcctccctccaattcttttggtatatatccagcagtgggattgct  
ggatcacatggtaatttttaattttttgaagaatcatcatactgttttccacggcagcagacca  
ttttatgttcccaccaacagttcattctagtttctccacatccttgccaacacttgctattttct  
ctttttgacagtacccatcctaagtgtgaggtcctgtctcattgtgggttttgattcttgagg  
ctttttaagctttttgtttcattataatttttattggattacaaaagggaacacaggttaatttta  
tttggaactatgaaaaataataaaaaattatcttctcagaaaaatgattcttgtaacatttaagc  
tcagttaagctctctcactttctctcccttctctctctttgtacaacttttaaaaaatatagtag  
gggtgagactatatgtatctatactatagtaggggtgagactatatgtatccttcccttttctact  
taatctcatgccttgagtagctttccactttattaaaaatgtgatgccattcaattgtatagtaa  
atacatatatgtgaagcaaaacactgaaaactcttattctgggttccagcaagccatacctggaat  
gggtgaagcaggtagtttgcttggtgtgaacgtgttggtgaggcagctgccattgtgttggtgagt  
gggccacacgaacttgctctgttggtgtgtagacagtggtgtgctgatcctattaggaacagccaac  
gctttgtgtgagccacacacgggttctaagtgtttgcttctgttaactcagtgaaactcctcacaac  
tccatgacgggaatgcttaattatccccattttatagatggggcaactgaggtccaagagactac  
ataatttcccgaaggtcacacaggttagcagatggcagagcgggtcaggagtcaccatcttacc  
acgcagactgttttagccagagactctccggatctgctgtaggggacagaatacagctttatcgc  
cgcacctgtccaccaagatggccttagccacagagcttggttggttaacgtcctctttatgtgac  
aggaaactgtgctgatgggggtttctgaaggtacttctgctctttgtctcctggaagactgtgtct  
tcaggaatgtctctgacctgcccagagtgaaacggatgctgggaaccacagcctgcacacggc  
cttccctccaggactctgcgcacctctgtgctccacaggagacatgcaggtgctttctctcatga  
gctcaggctcctgggctgacagctctccgaagctcgtgggtgaggctcgggtctctaactgtgccac  
ttgcccagggcctctgttcacaaggttccccctgctctcgatcttgcatcacccttgaatttg  
aaatccagagcagcccactcagagaccagtgtaggaattagtgtccaggccacagatccaggga  
ctgggcacaaacatctgcctgttgagttaggaactgagctgtggccattggcaaaaaaggagggggt  
gagcatggctgtttcttggggagctaacattcactatcttgtctcctccctcaggatgtggggca  
atcaagttggggatgaaggagcaaaagccttcgcagaggctctgcggaaccacccagcttgacc  
accctgagggttaactgtggccctgctgtctccaggggccaacctgggtccctcccagctgctctagg  
tttgctgggggaagggtgattcgtgctcctaataagaagaggaatttgcatgtgtgattttccttac  
tcttgtaaaacctttctttgatgcataagaggccatctagtaaaagcacattcttctctttttta  
actttaagttctgggatacatgtagaagatgtgcaggtttgttacataggcaaatgcatgccatg  
gtgatttgctgcacctatcaacctgtcatctaggttttaagccctgcatgcattaggtatttgct  
ctaattgcttgccctcccttgccccccaccccccaacaggccctgggtgtgtgtgtgttccctccat  
gtgtccatgtgttctcattgttcaactcccacttacagtgagaaacatgcagtggttggttttt  
gttctgtgttagtttgctgagaatgatggtttccagcttcatccatgtgccagcaaaggacatg  
atctcatttttttttatggttgcatagtatccatagtggtgtatgtgccacattttctttatcca  
gtctatcactgatgggcatttggttggttccaagtctttgctatttgtaaatagtgtacaataa  
acatacatgtgcttggtgtctttatagcagaatgattttataatcctttgggtaaataccagtaat  
gggattgctgggtcaaatgggtattttctggttctagatccctgaggaatcaccttaagtgtttatt  
cagctcagtggaattctgcattgtgtccacacagcccaaccaccccatcaagacagaggaca  
tttccagccctcagccatccctgcatgtcccttgctggtagaggagggttccctaagtgcaga  
tgaaacttaataagatgctggccagcagattcctgccccttcttgctcctcaggatgatgctgga  
aaagagggactcttctctctataaatggggatgcacctaccagcccccgcttaggctgctggc  
caaatcttgggaccttggtatgtccacggctctgctgctgttcttctaccactgaaaaagagtc  
caagaaggtggggacagtagcagaagagacttgccaggtcttgagatggggtagcttgatggg  
gccagcctttagaaggacagcttgccaggcctcgccagcctcctgccatgtgcagaaacctgag

gtgccgacccccagccccactgttgtgtgagcaggctgtgctgatgacccatttcccgtccagcctg  
cccttgtgctctgtgtgtgggctctggggcagcagcgcctgggcactactgctgcagctgaacac  
ttctgcatcctgccccgagtgagcctgggctggggccacagccaggcagaggcttcccagctgtt  
ctgatgttgaagctaagattgaatgtagatgtgtctttaataattcaccccaagtggtgttcttc  
ctagtcttgctccaacggcatctccacagaaggaggaaagagccttgcgagggccctgcagcag  
aacacgtctctagaaatactgtggtaatagctcgagtcatttcatttgtttgtttgttttctgt  
gatagggtcttgcttgttgcgtccaggcttgagtgcattgggtgtgatctcagctcactgcagcctc  
cacctcccaggctcatttcgaacctcccgcttggccttccgagtcctgagactataggcatgcac  
caccacacccagtttaatttttaaaattttttgttagagatgggggttttgctatgttaccaggctgg  
tcttgaactcctgggctcaagcagttctcctgccctggcttctcaaagccctgggattgcagggtg  
tgagccactgcacctggcacagagtcattttggagggttttaggtcccaggaattatcccaggggc  
tgcacatggcctggaatcttaacagaaaaagggtgtctcccaattggaaaggctctaggcctttcag  
ttaagtgtgataatttctcctagagaagagaatagccacttctacaagcataaacagggtacagga  
ggaggaagtgggctccgggagcctggatctgaggccttggccttctaggccccaggagaactaga  
acgctggccatgcaagctatccagggtatccttggataccttcagatgtgcttagcagaggccaac  
ttccacacacttggctcaaaattttctcccttccctcctcttcatctgccttccccaggcagcct  
cctccttccccaggctcttcacatcagggtttggcctttatgctccatccagctcatctgtcactt  
gtcacctgaagcccacagtcctcgctccctctctgcactctagggcacttactaagtggatgtgg  
cctcctgagagtggtttttgttgggtgttcccttttttatggccacttaatgttttattttgcttt  
atttgtatttacatctctgtatcataaattccatacagggtggctgggagcagtgactcacatctg  
taatcccagttcttggagggtgaggtgggaggatcgcttgaggccaagagttcgagactagcc  
tgggcaatatagcgagaccctctatctacaaaaaaaaaacattccttacaggtttaagtgggg  
agttgtattacaacctccctatcatctactcagagcccagtgctcatttgatcttgctaataa  
gttactgagaataatgacaatatcctcttcagagagagtttgacattaggcctgtgtccagt  
aagtgcaatttaatttcttccctcaacaaatcatttaacattttgaaaagtagtttatgtttt  
ttggaaaaaatgtaagacactaaaggaggacatgaaagtacctcctaaagtctcgtctaaaagga  
ggaagtgaagtacctccctttgtgttttccaaaataacctttcctttctagccttttgttctat  
gtatgttcaaagatatgcaaaacagaatagcattcaagcagtggtcttaaaaaatattgtaatcac  
atactttacatgtctcctttagggtttctccatcttgatgctgttgacatttttggtccaagtgat  
tctttattatggtagggctgtcctgtgcatcatagacggtttagccgcacatctctgccctgtacct  
cccagtggtgaggatcaaaaatgcctccggacatggccagggtgccccatggagagtgaaatcaca  
tggatagtagtaatgtcaacacctagaagccctcaagtgtgactgcattgccatgtgttattcta  
cacttttccctgtgttaactcactcagcctcacaaccactctatacgatctctactgtttaacgt  
tcaccagtgagaaaaactcagacccaaagaacttaagcctgttgcccagaggtcacccctgctgggtg  
gtgatataaaacctgcccaggctgagtcgggagtagatgtcaatgctgtgttcttctccctcctca  
ttctacctcatttctccctacaagctgcacaacatctcgaatagatatcacaatatatttcatcag  
ttgtttctgatctaaatttgttcagattttacattaggataataaccacaatgcattgctgcaatgt  
ataaagcctttgtgtgtatatccttgcacactgtagggttaatttctagaagtctgattgtcttaa  
aatgaagcacattaaaaatttgggcaggcacatccaaactgcccttcaaggaatttttttttta  
aatgttcttctgttctattcttcttccctaatgattcttctcgtccactggcacaagtgggtccta  
ccctgtttacaccaaggagctttgggtgctttatccagaccacttctgggtctaaaggaccattgag  
agacttctgaactttcagtcacttaacttgggtccctcacaagtttaactgagagcaaaagtactg  
aacacattttaatgtgcagtcagtgactgtttcagggtcttcaaactaaacttggataaacacactgt  
cagtggtgttcaagggaccctgggactagaggagaactgagaagcaggcattggccctttgtttt  
ccgtgggcccccatcttccatgaaatctgagggtcagcaagggtggggagggagggtgggctcc  
tctacaggtagctgggctaagaaataggagcccagggtacaggatttgcattaaaaaatgagtccta  
ttgaccttctgtggggctgacaggctgggcttggagcctggctgttttctgggttctcagcaagt  
gatcatctgcatagctggagagccttgggctgagctcccgtcctgtgaactctaaaacaatgtc  
tgccaagtaggctctcttgagtaataacttcttttttctttaggctgacccaaaatgaactc  
aacgatgaagtggcagagagtttggcagaaatgttgaaagtcaaccagacgttaagcatttatg  
gtaactcagagagccttacaatttcagactgtgctactttcaaaagtatttttgagataaaaat  
ttacatactgtaaaattcactctcttaaagtatacaatttcagagggttttttagtgcaacctacc  
acctaattctagaacattttcactcctcctccctcctcccaaaaagccctgggtatccattaaagcag  
tcactccctgtcctcctccccagaccctggcaaccactaatccgctttctgtctctatggatttg

FIG. 18 (7 of 10)

cctactctgggcatttcatataaatggaatcaagcaatatgtgaccttttgtctctgtgttctag  
catgtttcattcccttttatggctaaatgataattcactctaaggaaattttgcagtttattaat  
cagttgatgggacatttgggttgtttctactttttgactattatgcgtaatgctactgtgaacac  
tcctgttcatgcttttgggtgaacatatgttttcatctcttttgggaatatacctgggaatagaa  
tttctgggtcatatggcaattctgtaactttttgaggagccaccaaactgttttctaaagtggat  
gtactattttacattctcgccagcaatgtatgtggattccaatttctccacatcctcaccaacac  
ttattattgtccatcttttaaaatctagttatactagtggatgtgaagtaatttctgggttttga  
tttgcatttccctgatgacaacaatgttgaatgtctttttatgtgcctactgggagctgtatag  
cttctttggagaaatgtctccatatcctttgccatttttaaaattgggtttgtcttctaagtctg  
agttataggggttctctatatattctgggtgctagacctttactagatacaggttttgcaagtat  
tttctttctttctgtggagtttttctctttcttgatagtgcctttaaggacaacagttttta  
atttttgttttttttgagatggagtcttgctcttgccaccagacaggagtgcagtgccatgatc  
tcagctctctgcaacctccacctcctgggttcaagcgattcttctgcctcagcctcctgagtgt  
tgggattacaggcatcagccaccatgcctgtctcattttgtatttttaatagagatggggtttca  
ccatttaggcccaggctgggtcttgaactcctgacctcagggtgatccacctgcctcagcctcccaa  
agtgtctgggattacaggcgaaaagccactgcacctggccaatagtttttaattttgatgaagtc  
aatttatctatttttttctttgggttgccttgctgtcttctcagtgtcttatctaagaaatgattgccta  
atccaagatcacaaagaactccacctaaagttttctgttaagcggtatagttgtttccctcacat  
ataggtctgcaatccattttgagtttaatttttgtatagtgtaaagtgaggggttaacctcattctc  
ttgcacgtggatatccagctgtcccggcagcaccacgtgttgaaacagattatcttttctattga  
atggccttgacaccttgtcaaaaatcaattgaccataaatgtatgggtttatttctgaattctc  
tgttctgggtccattgatttatatgtctctcctatgccaggaccattgctgtagctttgtgtagta  
cattttgaaatcaggaggtgtgagttctactttgttcttcttctcaagattgttttagaccattc  
tgggttcttttgcatttcttatgaattcagactcacttgatcaatttctgcaaaaagactagactc  
tgctacatatgttttttctttccttttttagcctgcagaaattatttgatcccatccctaagtg  
aggccagcctctccaggagagcagagctaggacagggtcagaaagagaggtcttgggtgctttgt  
gcattcccaacctgcactggccctagtgaaggcagcccagtggtggatgtgcctggacactgc  
aggcttttttaggggcattaggtgctctccttctggcctcctgccacatcttgggtggaggctgc  
cttccctgccttcaaaaaagcctaagtgggtgactagaaaacagcagagtgtaactgaatacagaa  
cttgggtgccacttccctgggttctatttttgtcccttttgaaagggaagggtcattacctctgccat  
tgaaccagggggcccctagcccttgtggggtatggctgggagcaccagatcctgggtgcagcccag  
ccaccagtggtcctgtgtgttgggcagtaaacagtgaacagagctcccttccccctggacactgt  
gcctaataccctcctcttgaaatctcacacaccagtggtggggggcactcttatagttattct  
cagtttacagatgacacaactgaggcacagacagatgcgtttatttctcaaggttctgtagctg  
aacagtggggagggagggtttaagaggagctgcacccgctctgcaatactgcctctcagagggga  
gtcctcttcattcatgacagcatagggccctcgtcttctggtaagggttccctcttgggtcag  
tgccaggatttctaagggtcatgttttagcaggagcctattctacaaacagccaggagcagggat  
gactctgtgatgaagcggagacactacagcctcttgatgcatttatcttctgggtgggttagaag  
cgtagctgcccaggagcatttcaggagaggcctggcttctagcgatagctgaaaactttgtt  
tcatttgaatcactgctacccagaacaatgggggtgcattctcagagtccccattattaaagcttt  
tccactgagcccatgagaactattcatgagaactatttcatggcagcataactgtttctcctcc  
ctccctcttgcatgttggtagcctctttaaactttaaaacctgccttgccttccctagctacctgg  
aaggagacgtcagacttctgtcccatgggtgtgtttcttacaatttggtgttcagattggtggtc  
tcccaaatatataaaaaatataaatggagtctcactctgtcaccaggctggagtgcagtggtc  
cgatcttgggtcactgcaacctccacctcccagttcaagcaattctcctacctcagctctcccgag  
tagctgggagtagaggtgcacaccaccatgccagctaatttttttgtatttttaatagagacag  
gttggccaggatggtatcgatctcctgagctcgtgatccaccacacctcgccctcccaaagtgtg  
ggattacaggtgtgagccactgcacccggccccaatatttttgattatgcacctctgcagtga  
aatgcaaacacacacatcagttcatgtattacattatgttactataaaaaacaaacagaaaaatt  
aaaaaatatcaagctatcctttactctagtggatcttacctggacacttttagccagatacaaa  
tcacatggactcagttcttccctgaccaacttgtctcttatcccaaaacaccttgcaactccc  
ttacgaaggggtcaaatttgatccagttatgtgattttatacaagttatgttcttcttcaggc  
ttatccagaatcagatcacagctaaggggactgccagctggcagatgcgttacagagcaacact  
ggcataacagagatttggttaagatcccagcgtttgtcacagtaataacaccagtgactgtttact

FIG. 18 (8 of 10)

caccaccactgactgtgcaaggcacaacgcagggtgggtttctgtttattcctccagcaaccctgc  
acagtaatgggtattacctctgttttacagaggttagacagaggcccagaccagtgaataagggtg  
cccaaggctcactacgagagaagctagaattcagcccagaatgcctgattccatattctgtgctct  
cctgccctgggccccccgccctcatctaccttcattgggtgggatgggggaagtggccagtgaat  
gatttcctagtgggaagtaaatccccctgggactcagcaattgagagatgactgtgtggccagga  
gtttggagctcattctccccctttctgggttcgtaagacatttccaggctgacttgaactgac  
ctgtgctctttgtctacttctttttctgtcttgagaacttcttatgctaataagaagaaaaaa  
gtttgctttactgtgacattgagcgccatgccacttctttctgctcccataaggcacagacac  
tccccactcagcagctcccttaacaacttaattgcctgggtgacgtgggactgggtggatgctgg  
gagaggggcttattaactatgtcctcctttcatgactggggagaatttcatagccaattaaaaa  
aaaacaaaaaacagctccttggccaacacaggctcctcatacagtgttttttaactttgcttta  
gaacttgtttggaacttgtcataaaatcgatcagtttgggtgaattgcaaccaacaatataaaaa  
agaaaaacagaacagaacaaaaatatcaggatgcaatgtgcatgggtatgaaagtatcatttcattca  
tcttagttcatgcttgcatgtgagtggtgtgtgtttgcataagtgttggttcacaacataaaat  
gtaattcttatttaggggtgtagacaaaaagggttttttttaaaaaaacactgttggttaggcat  
gggtggctcatgcctgtaataaccagcacttgggaggccaagatgggcagatctcttgagcacagg  
agttagagaccagcctgggcaacatgcgaaaccccgctcactacaaaaattagcccgacatgggtgc  
tatgtgcctgtagtcccagctactcaggaagctgatgtgggaggatggatgcatgggagatcaag  
gctgcagtgagccaggatcatgccactgcaatccagcctgggtgccagagaccctgtctcaaaaa  
acaaaaaagaaaaaaagaaaaacaccatcatagagaatagagcccagatctaacagacacctgt  
ggcctgtgtgcctgcgaagcccagcctgccagcagcctgggaagcactggaggggcactggaact  
gtttgcatgggtgtttgccctcaggccactccgtttctgctgattcttaagtttgaggacagca  
ggcagagggggagaggaaggagactggcagactacagaacagtttgagagcacagttggcttcc  
actttctctgtagctgggtcaggcggttagtaaacagcttctgtgaggctatctcctccagactgtggctct  
caaaatctgcattgtctccctcttgagggtcaccattcctacacaaggaaccattttagtagggc  
caggagacttcagcttcaaggcctgcacttgtgtcagggtggagaggggaactggccaccaattc  
agagaggggcaggacaggcggtatgggtgctgggtcttgaggagtgtcttacttaggtccctggctt  
gttctgggagcctccagagcatgctcctctgtgtgtgacttcatgggactgggctctgagaaggc  
tgtggctttgttggccctgccagggactgccacaccaggccacagggttgtgggttgagctggccg  
gggagccacgttcaggggagcagctctgcttgagaccaacacttacagagtaagccttctccttg  
acttgttaactgtactgacacttatttctacctcattcctttctgaaaataacttggaagtctga  
agtcctttagatgagttctgtctttaagaacagaaaattagagggtgaacaatgaacactgtaattta  
cagaaatgtatcccaactccagataaacagcttctgtgaggctatctcctccagactgtggctct  
gggagggtggggcctgagtcagggtcctagggactagtgtgtgtcttcatatttcttgaata  
acgaaacgcttgagcatcagggactgtgctagcaccaaaaatccagtggtgaacaacatggcttc  
atgggttactgtctagaaagggagaagcacattaaagaaaaaatcatttgcgtaattattta  
tacaactgtgatgggtactatcacaaagggggaaggccaagaggggaacctgatttagatgaggttg  
cagggaaggcctctctgaggaagcagcacttacactaagccatgaaggatgaataggagctagtc  
agctgaggtgagatattctgcgtagggaacagcatgtgcaaagggtctggggcaggagggagtgtg  
gtgtcctggaagaactgccagaagctgctgtgccccagggttcagacagtgtggaagaggggact  
acaggaggctgaggagataggcagggactggaccataaaagatctgtgggtcatgatgtgcattt  
tggtctttatcctaaaaagtgtatggaaagtcagtgaacagtttgaagcaggagaggcatgtgatca  
gatctgcaatgcaaaaagaccaattcttggtcttctaggaaactgaattggagaaggccagagt  
acgtggaaatgacctgtcagtaggacatttgtactgatgcagggaagagatgatgggtgctcagac  
caagatggccggccaaagacatagaggttccaggggaggcattctagattcttaggaattagggga  
gaactttgtgatacaaggaacatggggatgagaagggaagggtgtccaggttgacccccagggtact  
aacctgctcagcaggatgagagtggtccattcactaagccaggggaccctaggaggtgtggctac  
tttgaggtgtgggggagaggtccaagtgaggatgccaagcaggttaactgcctccacggacataca  
aacaaggccgtggcattgatgagatcggttggggaaaagggttagcccaaacctggaggaaat  
ctcagatgtagaggtcacatggaggagaatataggaaaggaaattgaagtagagtgtcagatgc  
aggagaaaaatcagcgcatataaccaagccaaaggggagggtgctcaagaaggaggaggagga  
gaggtcaggacagccaaaatcctgaggggccaagaaagacaagacctggaaaatgcttaaatc  
aggcttatggagggtacaggtgaccttagtgagaccagtgaaacagagggtggcagctggagag  
gatccatgctaataatgaaggaaactatctgcaaagggtatgttccttaatttcagggatacatgtg

tattgtgtgatacacgagtgtgtgtgctatgaacacaccttgggaaggagtggtgcgaggatccttaa  
cattttacctgtgtacttttgtcttcctccttttcaacagcctaaatggaaacctgataaaacca  
gaggaggccaaagtctatgaagatgagaagcggattatctgtttctgagaggatgctttcctgtt  
catgggggttttggccctggagcctcagcagcaaatagccactctgggcagtcttttgtgtcagtgt  
cttaaagggggcctgcgcagggcgggactatcaggagtccactgcctccatgatgcaagccagcttc  
ctgtgcagaaggctctggtcggcaaactccctaagtaccgcgtacaattctgcagaaaaagaatgt  
gtcctgcgagctgttgtagttacagtaaatatactgtgaagagactttattgcctattataa

FIG. 18 (10 of 10)



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/02544

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07H 21/02, 21/04; C12N 15/11; C12Q 1/68; C07K 5/00  
US CL : 435/6; 536/23.1; 530/300

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/23.1; 530/300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONEElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
APS, MEDLINE, BIOSIS, EMBASE, CAPLUS, SCISEARCH

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HOFMANN et al. The CARD Domain: A New Apoptotic Signalling Motif. TIBS. May 1997, Vol. 22, pages 155-156, see entire document.	15, 17-19, 21-22
A,P	McCARTHY et al. RIP2 is a Novel NF-kB-Activating and Cell Death-Inducing Kinase. The Journal Of Biological Chemistry. 03 July 1998, Vol. 273, No. 27, pages 16968-16975, see entire document.	15, 17-19, 21-22
A,E	YAN et al. mE10, a Novel Caspase Recruitment Domain-Containing Proapoptotic Molecule. The Journal Of Biological Chemistry. 09 April 1999, Vol. 274, No. 15, pages 10287-10292, see entire document.	15, 17-19, 21-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

19 MAY 1999

Date of mailing of the international search report

26 MAY 1999

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

SEAN McGARRY

Telephone No. (703) 308-0196